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THE DEVELOPMENT OF HORIZONTAL CANALS IN RAYS

By M. MARGARET CHATTAWAY*

[Manuscript received August 23, 1950]

Summary

The development of horizontal canals has been studied by means of serial tangential sections in three genera of non-pored (gymnosperm) and six genera of pored (angiosperm) woods.

Horizontal canals, which occur in the rays of some genera of woody plants, originate as the result of changes in the cambial layer and are always developed in conjunction with vertical canals.

In the genera studied, vertical canals were found only in the xylem in non-pored timbers and only in the phloem in pored woods. They result from changes that either affect the cambial initials temporarily, or affect only the daughter cells, for subsequent divisions of the initials produce once more the normal tissue sequences of xylem or phloem.

The horizontal canals result from the same stimuli, and their origin is thus different in the two types of wood; it is in the vertical canals of the xylem of non-pored woods and in those of the phloem of pored woods. The changes that produce the horizontal canals affect the ray initials themselves permanently, for once the canals are formed they are continuous through the rays in both the xylem and the phloem that is subsequently produced.

I. INTRODUCTION

The presence of radial canals and secretory tubes in the rays of wood has, for many years, been considered a reliable diagnostic feature for purposes of identification; they are seldom traumatic, but are a normal feature of the woods of certain genera, and it is therefore strange to find that the information about them is very scanty. Their presence has been referred to time and again, but few references can be found to any work on their origin or development. Such as can be found is largely the result of investigations on young stems, leaves, and leaf traces. A resumé of the early work is given by de Bary (1884) and it appears that little has been done since, except on some genera of the Coniferae. De Bary mentions that the great majority of the Coniferae have no resin ducts in the primary tissues of the stem except those found in the cortex, and these connect only with those in the petioles and leaves. He mentions, however, that in species of *Pinus*, *Picea*, *Larix*, and *Pseudotsuga* there are also canals that are not continued into the leaf, and that these occur in the xylem of the primary bundles. It is significant that these four genera alone have resin canals in the rays. The work of Thompson and Sifton (1925) and the investigations recorded below show, however, that vertical canals are not always present in the

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primary wood, or even in the innermost ring, in species of *Picea* and *Pseudotsuga*. When this occurs the horizontal canals too are absent from the primary rays and make their appearance only in connection with the innermost vertical canals.

The work that has been done on secretory passages in angiosperms is equally far back in botanical history, and has mainly been carried out on small branch material, with the greatest emphasis laid on leaf trace and petiolar bundles and the structure of the outer cortex. There are few references to any work on the mature wood. Such references, however, as are available bear out the evidence that radial canals originate in the secondary phloem of angiosperms and not in the wood.

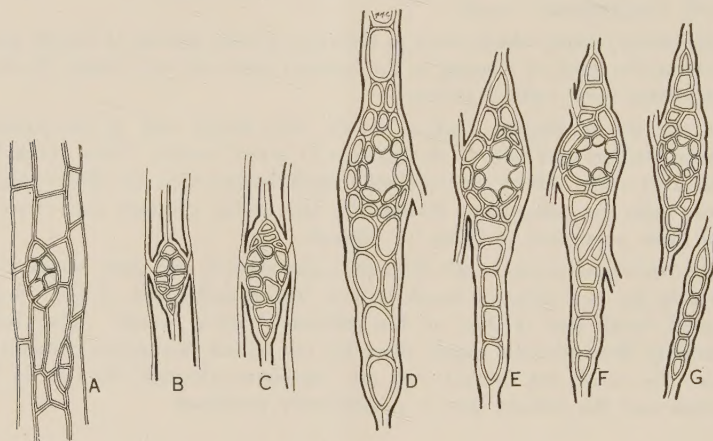


Fig. 1.—*Pinus radiata* D. Don.

A-C, tangential sections of small fusiform ray developing from primary medullary parenchyma. x120.

D-G, later stages in the development of two rays. x120.

Much of the early work was done on the predominantly herbaceous families such as the Compositae, but de Bary quotes work done by Trecul in 1886 on the Anacardiaceae. Trecul described "gum-resin-passages" in the primary and secondary phloem of genera of the Anacardiaceae, but suggested that the canals in the rays arise as "blindly ending branches which penetrate here and there horizontally into the medullary rays of the xylem." This picture of branching canals which actively grow into the fully formed rays is, of course, totally incorrect, and it can now be shown that the "blind ends" of Trecul are each the beginning of a newly developing canal. The connection of the radial canals with the vertical ones is correctly described, but the two develop simultaneously in the cambial region under some stimulus that changes the initials of the cambial layer itself. By the persistence of this change these canals must develop afterwards in the xylem as well as in the phloem, and, owing to the greater number of cambial divisions that occur towards the pith, extend a greater distance through the xylem than through the phloem.

II. MATERIALS AND METHODS

The material used represents eight genera of three families of pored timbers and three genera of non-pored timbers:

Coniferae	<i>Pinus radiata</i> D. Don, <i>Picea smithiana</i> Boiss., <i>Pseudotsuga taxifolia</i> (Lamb.) Britt.
Anacardiaceae	<i>Euroschinus falcatus</i> Hook. f., <i>Microstemon velutina</i> Engl., <i>Pistacia lentiscus</i> L., <i>Pleiogynium cerasiferum</i> Domin.
Burseraceae	<i>Garuga floribunda</i> Decsne., <i>Protium australasicum</i> (F. M. Bail.) Sprague.

Sectors of wood were cut so that the same rays could be traced for several centimetres, starting if possible from the pith, and these blocks were cut into serial tangential sections of 35-40 μ thickness. By this technique the rays can be identified in the narrow sections of the young wood and then followed in successive sections of the series and their growth noted as they keep pace with the expanding circumference of the stem. This method of tracing the origin of elements from the cambium by studying their appearance in the mature wood has been used by Klinken (1914), Neef (1920), and Beijer (1927) in studies on the stratified cambium that gives rise to storeyed structure and ripple marks in wood. The technique depends on the fact that the secondary thickening of the cell wall, which occurs during the differentiation of woody tissues cut off from the cambial layer, causes a rigidity of the cells, and, as soon as it is complete, prevents any further alteration of shape. Thus the shape of the wood cells at any given point in the trunk of the tree, as seen in tangential section, gives a very fair picture of the state of the cambial layer when the cells were cut off. Extra-cambial changes may produce differences between adjacent cells in the wood — as for instance between fibres and parenchyma cells in the same radial series — but such changes are easily recognized, and the structure of the wood as seen in a series of tangential sections gives a picture from which it is easy to reconstruct the state of the cambial initials when that wood was laid down. As the rays do not grow tangentially or vertically after differentiation, this method has proved to be singularly well adapted to their study, and has been used to follow the development of the rays of the Sterculiaceae (Chattaway 1933a) and of the tile-cells in the rays of the Malvales (Chattaway 1933b). More recently it was used for a study of the vascular strands that occur in some of the rays of *Banksia* and *Dryandra* (Chattaway 1948). It has now been applied to an investigation of the origin of horizontal canals.

III. OBSERVATIONS

(a) Resin Canals in the Rays of Primary and Secondary Wood

(i) Non-pored Timbers (Gymnosperms)

Pinus radiata.—Tangential sections from the pith outwards showed that the primary medullary rays develop as loosely organized masses of parenchyma

that gradually subdivide into smaller and more compact masses and soon become recognizable as the small uniseriate rays characteristic of the secondary wood. They are later interspersed with multiseriate rays containing radial resin canals. The origin of the radial canals is near, but not in, the pith; they are not present in the loosely organized mass of the primary medullary rays, but begin to appear very soon after the secondary rays are recognizable. Figure 1A-C shows a small ray containing a resin canal developing within a primary parenchymatous mass. In Figure 1D-G, later stages are shown in the development of two rays. The large, loosely arranged ray seen in Figure 1D becomes more compact in E and F, and in G is subdivided to form a multiseriate ray containing a resin canal and a uniseriate ray.

It is difficult to follow the origin of these large primary rays from tangential sections alone, as the loosely arranged parenchymatous tissue tears very easily under the microtome knife, but their development becomes quite clear when the tangential sections are examined in conjunction with cross and radial sections of the pith and early wood.

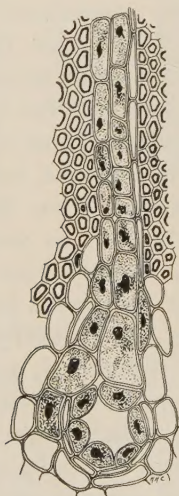


Fig. 2

Fig. 2.—*Pinus radiata* D. Don. Cross section of inner wood, showing a multiseriate ray developing in connection with a vertical resin duct. x85.

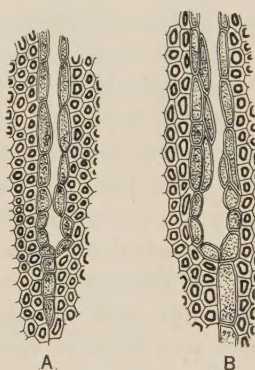


Fig. 3

Fig. 3.—Cross sections of the secondary wood showing uniseriate rays that become multiseriate in connection with a vertical canal. x120.

A. *Picea smithiana* Boiss.

B. *Pseudotsuga taxifolia* (Lamb.) Britt.

There are no vertical resin canals in the pith of this species, but an almost complete ring of vertical canals occurs within the ring of primary wood (Plate 1, Fig. 1) and it is from these canals that the radial canals in the first annual ring originate (Fig. 2). As the rays diverge with the increasing girth

of the tree, new secondary rays are formed. These are always uniseriate. The origin of new fusiform rays is always in connection with a vertical resin duct, resulting from changes in the initials of existing uniseriate rays that were contiguous with the initials from which a vertical canal was formed and were therefore subjected to the same stimulus.

Picea and *Pseudotsuga*.—The origin of the horizontal canals in *Picea smithiana* and *Pseudotsuga taxifolia* was found to be similar in detail to that observed in *Pinus radiata* (Fig. 3A, B; Plate 1, Figs. 2 and 3), though the almost complete ring of vertical canals was absent from the inner wood of both, and there were no vertical canals in the first annual ring of the specimen of *Picea* examined. This may possibly be due to the material having been a branch from a tree from the Botanic Gardens, Melbourne, for Thompson and Sifton (1925) have commented on the absence of vertical canals from the first few rings of *Picea* sp. grown under protected conditions. The descriptions and plates given by these authors show that the development of the radial canals in Canadian spruce is identical with that noted in *Picea smithiana*.

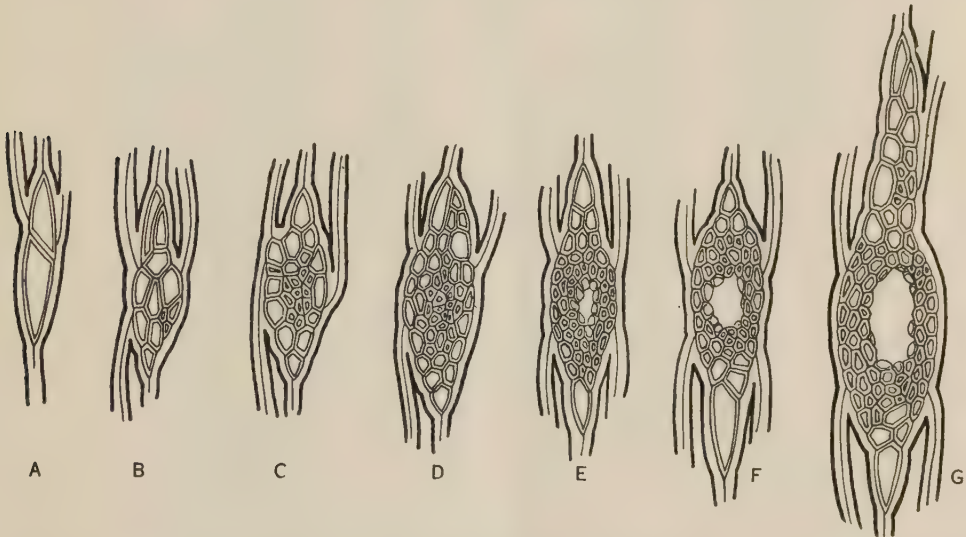


Fig. 4.—*Euroschinus falcatus* Hook f.

A-G, development of horizontal secretory canal through approximately 2.5 cm. of wood. x175.

(ii) *Pored Timbers* (*Angiosperms*)

Euroschinus falcatus (Anacardiaceae).—Material was available for study of this species from the pith outwards through several inches of wood. In the innermost sections, which were in the primary wood, all the rays observed were uniseriate, some of them very high, but all were seen to break up within about 0.5 mm. of the pith into uniseriate rays that varied from a few to about 30 cells in height. No radial canals were found in these rays. A few biseriate rays were developed in the first millimetre or so, and by about 3 mm. from the pith two radial canals were noticed; it was not possible to follow these

back to their inception. At this distance from the pith most of the original rays had become biseriate and several of them were found to be developing radial canals, which could be followed in subsequent sections. Figure 4 shows details of their development. In all the rays of *Euroschinus* examined that showed resin canals in their later stages, the inception of the ray was no different from that of any other newly formed ray. However, subsequent divisions produced, not the normal biseriate, or occasionally triseriate, ray which is found throughout the mature wood, but a group of small cells the walls of which ultimately separated to produce a cavity. The details of this can be seen in Figure 4A-G. The actual cavity into which the secretion is poured from the surrounding cells is schizogenous in origin, the cells apparently separating along the intercellular layer. The secreting cells lining the cavity seemed little different from others of the surrounding sheath, except that their walls were somewhat thinner than those of the rest of the ray, and curved markedly into the cavity of the secretory passage. These cells are apt to tear on cutting and cannot all be observed in any single section.

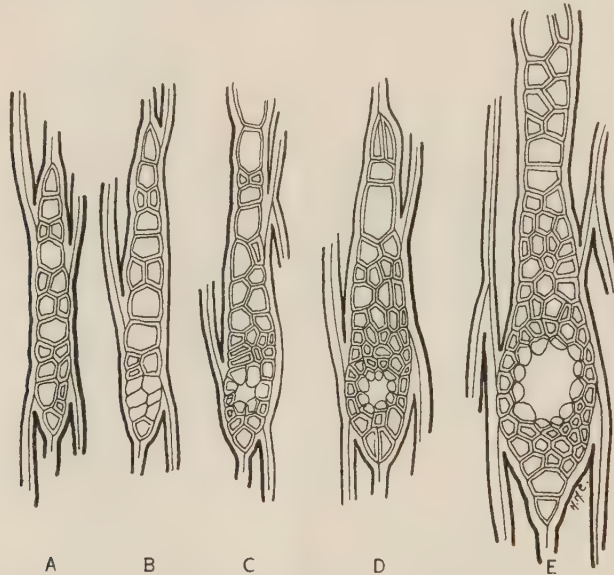


Fig. 5.—*Pleio gynium cerasiferum* Domin.
A-E, development of horizontal secretory canal through approximately 4 mm. of wood. $\times 175$.

Pleio gynium cerasiferum (Anacardiaceae).—Fresh branch material of this species was available, and it was again possible to trace the rays from the pith outwards.

The primary rays were found to be predominantly uniseriate and there were no secretory passages in the innermost millimetre of the wood. Secretory passages appeared first in the secondary wood about 1 mm. from the pith, as schizogenous passages between the cells of biseriate rays, enlarging as the rays

increased in size. The course of development, which is fundamentally similar to that in *Euroschinus falcatus*, is shown in Figure 5. The main detail of difference appears to be the development of the thin-walled cells at an earlier stage in this wood. *Pistacia lentiscus* (Anacardiaceae) and *Microstemon velutina* (Anacardiaceae) showed very similar ray development. The details can be followed in Figure 6A-G. The small ray cells in *Pistacia* are due to the sections having been cut from rather small branch material.

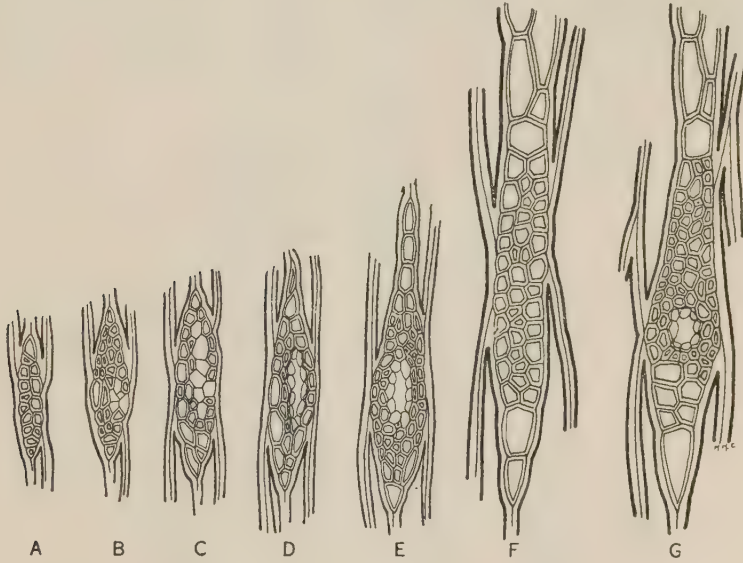


Fig. 6

A-E, *Pistacia lentiscus* L. Development of horizontal secretory canal through approximately 6 mm. of wood. x175.

F,G, *Microstemon velutina* Engl. Tangential sections before and after a horizontal secretory canal has developed. x175.

Garuga floribunda (Burseraceae).—The pattern of development in this wood followed the same general lines as that in *Euroschinus falcatus*. It was not, however, possible to procure material for studying the rays at the pith, but serial sections of the secondary wood showed that the secretory passages started through subdivisions of the existing ray initials, as in other woods studied. The stages of development are shown in Figure 7. The thin-walled tissue that occludes the cavity of the canal in the final diagram must not be confused with epithelial cells. The sections of this wood were cut in the heartwood, and the cavity of the canal has become filled with outgrowths from adjacent ray cells—tylosoids (Record 1934).

Protium australasicum (Burseraceae).—In this wood the details of development were essentially similar to those already described, the details can be seen from Figure 8A-J.

(b) *Resin Canals in the Rays of the Primary and Secondary Phloem*

Unfortunately the phloem is not such a satisfactory medium for the use of the serial tangential section method of following ray development. As the cambial initials cut off more cells towards the wood than towards the phloem the wood rays can be studied through many inches while phloem of the same age would fail to provide as much centimetres. The difficulties are further increased by a tendency of most trees to cut off successive parts of the phloem by the development of phellogen or cork cambium, and by the inevitable crushing of the soft outer tissue as the more rigid central core increases in diameter.

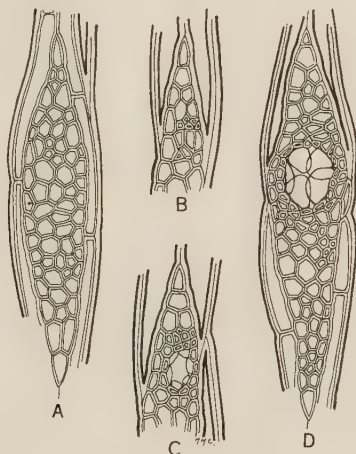


Fig. 7.—*Garuga floribunda* Decsne.
Development of horizontal secretory canal through approximately
1 cm. of wood. $\times 120$.

Pinus radiata.—Serial tangential sections of the phloem were cut from the cambium outwards so that the rays were followed in a reverse direction from that taken in the wood—from the fully formed ray out towards its inception. For the reasons mentioned above it was not easy to find the earliest stages of the development of the canals, but sufficient were seen for it to be clear that they paralleled very closely those observed in the wood rays of angiosperms. The canals developed apparently spontaneously without any connection with vertical ducts. Cross sections showed that vertical ducts were absent from the phloem of *Pinus radiata*.

Pleiogynium cerasiferum and *Pistacia lentiscus*.—Transverse sections of the stem of young material showed that though vertical canals were absent from the wood of this species they were plentifully developed in the primary and secondary phloem, and that it was in connection with these vertical canals that the medullary canals in these species originated. Figure 9 shows the origin of one such radial canal in *Pleiogynium cerasiferum*. In many instances

the connection of the ray and the vertical canal could be seen, though the sections did not always pass through the canal itself as is shown in the figure.

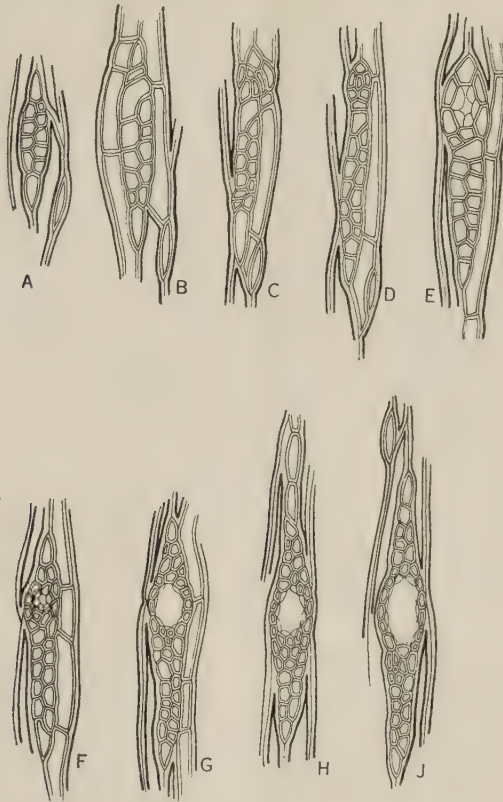


Fig. 8.—*Protium australasicum* (F.M.Bail.) T.A. Sprague. Development of horizontal secretory canal through approximately 1 cm. of wood. $\times 120$.

Tangential sections were cut from the cambium outwards in both these woods, as in the material of *Pinus*, and the resin canals in the rays traced as far as possible. Owing to the crushing and to the parenchymatous nature of the outer phloem, the rays become ill-defined and very different to follow, but the canals could be traced without any noticeable diminution in size through the phloem, and in one or two instances were observed to be in distinct connection with the vertical canals. This supports the clearer evidence of the cross sections (Plate 1, Fig. 4) that there are vertical canals in the phloem with which the radial canals in *Pleiogynium* and *Pistacia* can form a connected system.

IV. DISCUSSION

From the foregoing observations it is clear that there is a very fundamental difference between the development of horizontal canals in pored and non-pored timbers. In each type of tree there is a system of vertical canals

with which the horizontal canals are connected, but whereas in non-pored timbers this connection is found in the wood, in pored timbers it is found in the phloem.

Neither the vertical nor the horizontal canals can arise in the mature wood; they are the result of changes that have affected the cambial initials. Thompson and Sifton (1925) have stated that, in non-pored timbers, the formation of vertical canals is always the result of injury to, or irritation of, the cambium. But, whatever their cause, these vertical canals are clearly the result of some stimulus that has permanently affected the daughter cells and not the fusiform initials, for the vertical canals in non-pored timbers occur in the wood only, and the fusiform initial from which they arose subsequently produces the normal vertical elements of the wood. A horizontal canal appears to be formed when

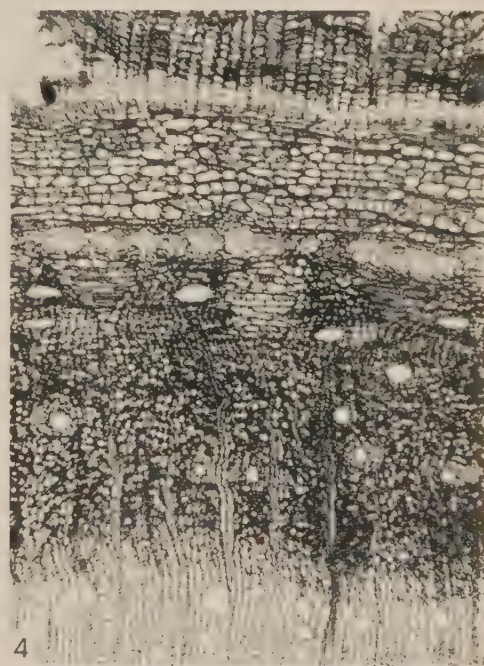
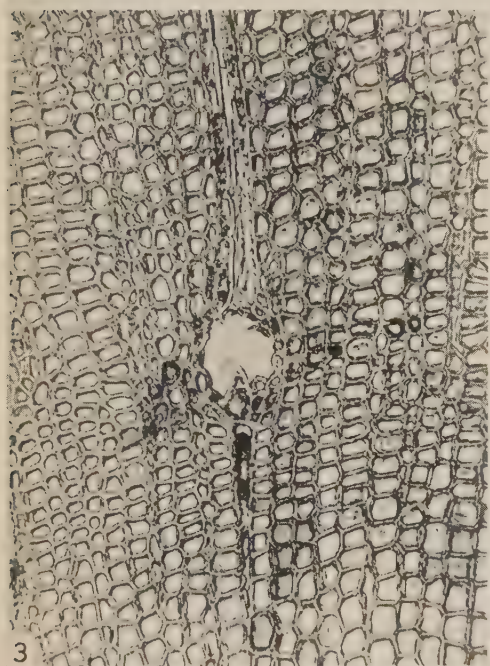
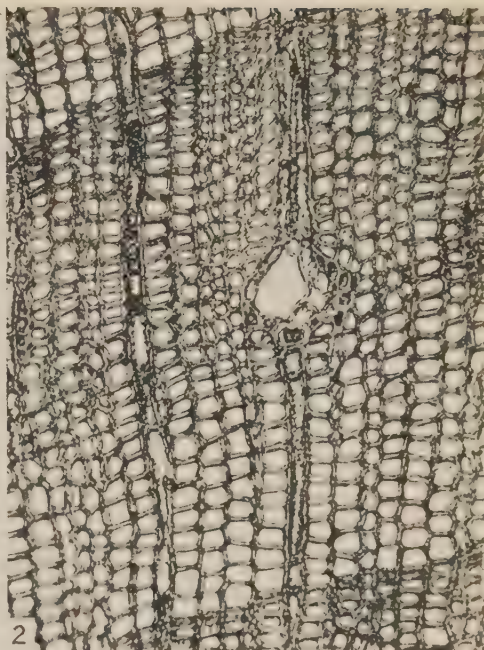
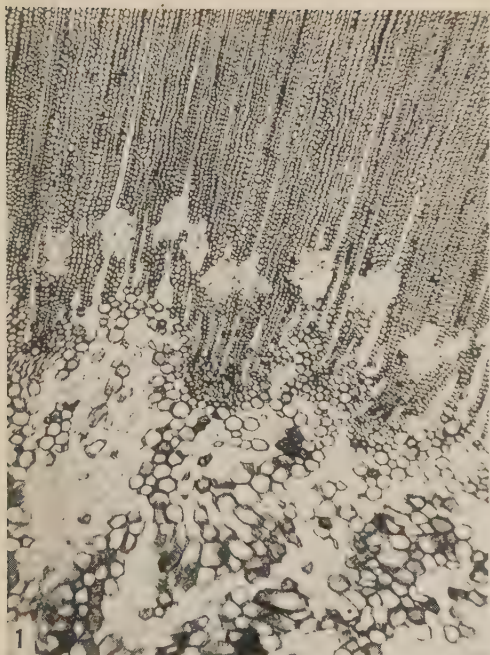


Fig. 9.—*Pleio gynium cerasiferum* Domin. Cross section of vertical secretory canal in the phloem, showing the origin of a horizontal duct from the vertical one. $\times 120$.

a ray initial occurs in the area of the cambium that is stimulated to form a vertical canal. Plate 1, Figures 2 and 3, shows uniseriate rays that have become multiseriate after they have passed around or through a vertical canal. Clearly the ray initials and fusiform initials in this area have received the same stimulus. Yet, with the fusiform initials only the daughter cells have been affected, but in the other the actual initials themselves have undergone a change. For the horizontal canal occurs in all the ray tissue subsequently produced, whether in the xylem or phloem.

In pored timbers the position is reversed. In all the material examined the vertical canals are developed in the secondary phloem and not in the xylem. The origin of the horizontal canals is again to be found in connection with the vertical ones, but the stimulus that causes production of these affects the outer daughter cells of the cambium. As in the non-pored timbers the actual ray initials are changed in the formation of the horizontal canals, which are continuous from phloem to xylem.

HORIZONTAL CANALS IN RAYS



Small vertical canals, similar to those described above, occur in relatively few genera of angiosperms, and have been found associated with horizontal canals in one genus only. They have been reported in conjunction with radial canals in one subsection on the genus *Shorea* (Desch 1941). Unfortunately it has not yet been possible to obtain material of the pith or phloem of any of these species, and the material available was not suitable for study by the serial tangential section method. However, no connections between horizontal and vertical canals could be seen in slides of the xylem. It would be interesting to know whether, in these species, as in the pored timbers investigated, canals are present in the secondary phloem.

V. ACKNOWLEDGMENTS

The author wishes to express her thanks to Dr. H. E. Dadswell for advice and encouragement throughout the work, and to the Director of the Botanic Gardens, Melbourne, for fresh material of some of the genera studied.

VI. REFERENCES

- BEIJER, J. J. (1927).—*Rec. Trav. Bot. Neerland.* 24: 613.
 CHATTAWAY, M. M. (1933a).—*New Phytol.* 32 (4): 261.
 CHATTAWAY, M. M. (1933b).—*Forestry* 7 (2): 93.
 CHATTAWAY, M. M. (1948).—*J. Coun. Sci. Industr. Res. Aust.* 21 (4): 275.
 DE BARY, A. (1884).—"Comparative Anatomy of the Phanerogams and Ferns." (Eng. Trans.). (Oxford University Press.)
 DESCH, H. E. (1941).—Dipterocarp Timbers of the Malay Peninsula. *Malay. For. Rec.* 14.
 KLINKEN, J. (1914).—*Bibl. Bot. Stuttgart.* 84: 1.
 NEEF, F. (1920).—*Z. Bot.* 12: 225.
 RECORD, S. J. (1934).—"Identification of the Timbers of Temperate North America." (John Wiley and Co.: New York.)
 THOMPSON, R. B., and SIFTON, H. B. (1925).—*Philos. Trans. B* 214: 63.

EXPLANATION OF PLATE 1

- Fig. 1.—*Pinus radiata* D. Don. Cross section of pith and inner wood. x 20.
 Fig. 2.—*Picea smithiana* Boiss. Cross section of secondary wood. A uniseriate ray becomes fusiform after passing through a vertical resin canal. x 180.
 Fig. 3.—*Pseudotsuga taxifolia* (Lamb.) Britt. Cross section of secondary wood. A uniseriate ray becomes fusiform after passing through a vertical resin canal. x 180.
 Fig. 4.—*Pleiogynium cerasiferum* Domin. Cross section of phloem showing vertical secretory canals. x 65.

MORPHOLOGICAL AND FUNCTIONAL VARIATIONS IN THE RAYS OF PORED TIMBERS

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Summary

The rays of pored timbers may be made up of cells of very diverse forms and functions and may form a horizontal tissue system as varied as the vertical system of the wood.

Differences in structure between ray cells are described and their development from the cambial initials is discussed. A distinction is drawn between changes that occur in the ray initials themselves and those developing during the differentiation of the daughter cells.

The influence of cell contents on the shape, size, and pitting of the ray cells and the reversible nature of changes affecting the cambial initials are discussed. It is suggested that the stimuli causing the change from one type of ray initial to another are as likely to be found in the phloem as in the wood and that in further studies of ray development more attention must be paid to the phloem.

I. INTRODUCTION

The intensive anatomical work that has been carried on during the past 20 years has shown that the simple concept of ray structure based on differences in cell shape is totally inadequate to describe the numerous variations found in woods that are now in use, or to aid in the identification of unknown timbers. Furthermore, though the rays in the majority of dicotyledonous woods are composed entirely of parenchymatous cells, there are many exceptions and ray tissue can include a variety of cells and even tissues of very varied functions. It would seem that the time has now come to take stock of our knowledge of ray structure and to try to find out what differences there are in function between the different cells of a ray, whether these differences are due to the position of the cells in the ray, and how far the cell contents play a part in determining the shape of the ray cells.

It has recently been necessary to make an intensive study of the rays of many different woods, both from a morphological and from a physiological standpoint. This work has shown that, although the rays of many woods may be composed of cells which are all of the same shape, such rays are not necessarily functionally homogeneous. Furthermore, there is a functional heterogeneity not only between the different files of cells that compose a ray, but even within a single file of cells, depending upon the different vertical elements of the wood with which the individual cells of the ray are in contact. Lastly,

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multiseriate rays differ within themselves, for there is a difference between the peripheral cells in contact with the vertical elements of the wood and the central cells, which are in contact with other ray cells only.

The work of Kribs (1935) and Barghoorn (1940) and others has shown that a phylogenetic sequence can be traced through the different ray types in related woods, and that a similar sequence may often be found from the pith to the mature wood of any single tree. In every case the cell shape in the fully differentiated wood mirrors the conditions that existed at the periphery of the stem when those cells were differentiated from the cambial initials, so that the whole sequence from pith to bark gives a picture of the cambial changes that have taken place during growth from the seedling to the mature tree.

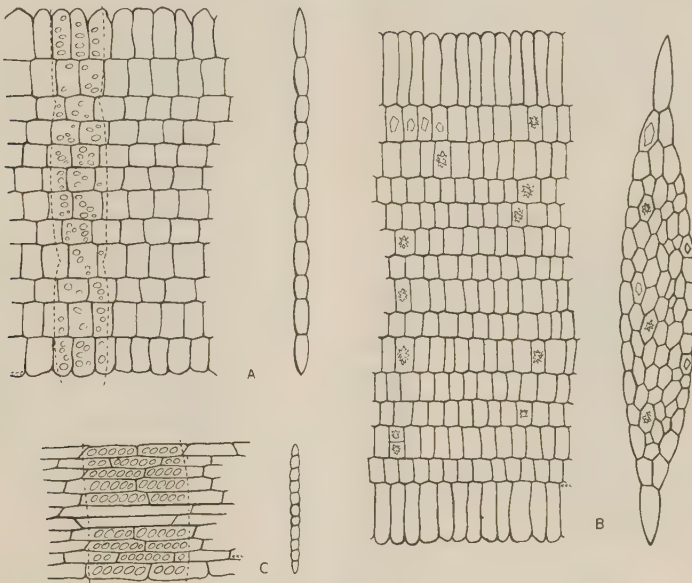


Fig. 1

A, *Guilfoylia monostylis* F.v.M. (Simarubaceae).

B, *Pipturus argenteus* Wedd. (Urticaceae).

C, *Eucalyptus regnans* F.v.M. (Myrtaceae).

(All $\times 85$.)

In order to understand the part played by the rays during the life of the tree, three different phases of ray activity must be borne in mind. Firstly, that cell shape is determined very soon after the ray cells have been cut off from the ray initials in the cambium; secondly, that certain metabolic by-products are laid down in the cells at this early stage of ray development (silica, crystals, etc.); and, thirdly, that as long as the ray cells contain living nuclei, they are capable of metabolic activity. This may show itself by the fluctuating cell contents (sugars, starch, etc.), or by excessive growth into the only available spaces (tyloses into the vessel cavities) under some new stimulation at a late stage in the life of the cell. It is the interaction of these three phases of cell

growth that gives the final pattern to the heartwood timber, and the present paper is an attempt to make clearer the interrelation of these three phases of growth, and their significance in the life and development of the tree.

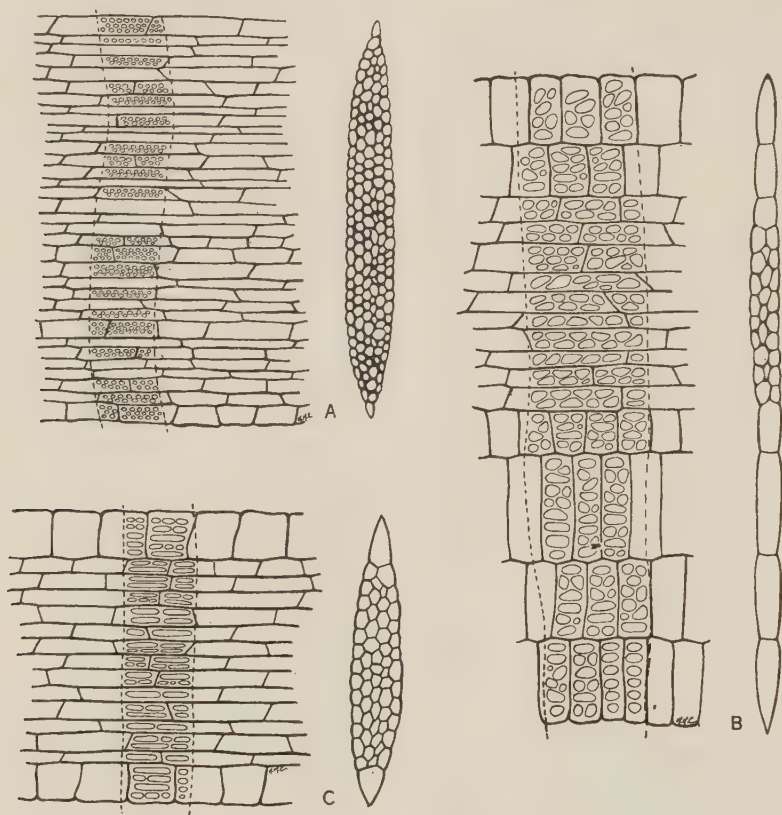


Fig. 2

A, *Acer pseudoplatanus* L. (Aceraceae).

B, *Endospermum peltatum* Merr. (Euphorbiaceae).

C, *Panax elegans* F.v.M. (Araliaceae).

(All $\times 110$.)

II. OBSERVATIONS

(a) Cell Shape

In order to simplify descriptions of wood, rays have, in the past, been classified as homogeneous — composed of radially elongated cells — or heterogeneous — composed of cells of different morphological types, typically with the cells of the uniseriate parts vertically elongated or square and those of the multi-seriate parts radially elongated.

Such a classification is quite satisfactory when applied to many woods, but there are a great number that are exceedingly difficult to fit into any existing

definition of these terms. It is now some years since the terms were defined (International Association of Wood Anatomists 1933) and most workers on wood structure have found it necessary to qualify the original definitions (Kribs 1935; Reinders-Gouwentak 1949) and even to re-define the terms during the course of a single large work (Moll and Janssonius 1906-36).

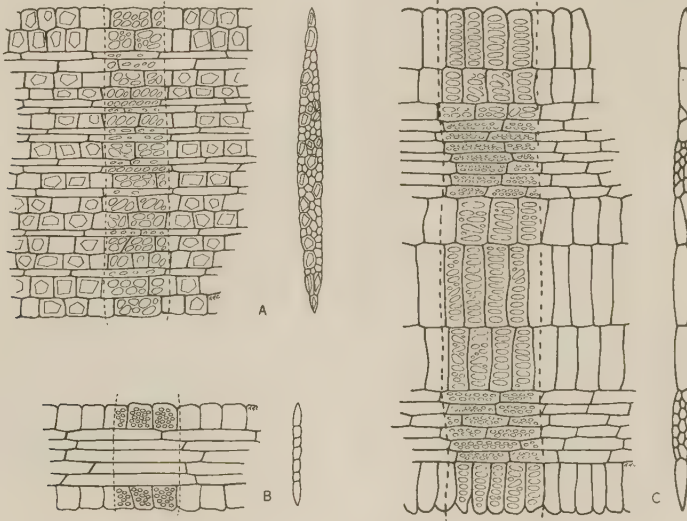


Fig. 3

A, *Hopea nutans* Ridl. (Dipterocarpaceae).

B, *Salix caerulea* Sm. (Salicaceae).

C, *Palaquium galatoxylum* (F.v.M.) H.J.Lam. (Sapotaceae).

(All x85.)

The proportions between vertical and radial dimensions have recently been measured in many different woods, and variations have been found between a vertical : radial proportion of approximately 10 : 1 in the erect cells, and a radial : vertical proportion of approximately 40 : 1 in the procumbent cells. Within this range almost any combinations of proportions may be found, from rays composed entirely of cells with greater vertical than radial dimensions (*erect* or *square* cells only), as in *Guilfoylia monostylis* F.v.M. and *Pipturus argenteus* Wedd. (Fig. 1A and B), to those composed of square or procumbent cells only, as in *Eucalyptus regnans* F.v.M. and *Acer pseudoplatanus* L. (Figs. 1C and 2A). Between these two extremes is a large range of different ray types, varying from those with high margins composed of many rows of erect cells, as in *Endospermum peltatum* Merr. (Fig. 2B), to those in which the ray has only a single row of erect cells on each margin, as in *Panax elegans* F.v.M. (Fig. 2C), or in which the erect and procumbent cells are intermixed, as in *Hopea nutans* Ridl. (Fig. 3A).

(b) Ray-Vessel Pitting

On examining different woods it soon became clear that even where the rays are homogeneous in respect of cell shape they may be very heterogeneous as regards function, and that even in a uniseriate ray, in which superposed files of cells are contiguous with the same element, there is often a very marked difference in function between these files.

This difference between the different files of cells in a uniseriate ray was noted by Kny (1909), who described a difference in turgor pressure between the marginal row or rows of cells and those of the rest of the ray in *Salix*, *Populus*, and *Castanea*, as well as a difference between the marginal cells that were contiguous with the vessels and those that were not. As these woods all have uniseriate rays, the cells of all the files in each ray are equally contiguous with the vessel. Nevertheless, it is only the upper and lower files in *Salix* (Fig. 3B) and the two or three marginal files in *Populus* and *Castanea* that communicate with the vessels by pits. Heterogeneity of shape may be present, but what is most marked and can be seen on both radial and tangential sections is that this slight difference in the shape of the cells is accompanied by a marked difference in function. The pitted cells must function in the interchange of solutes between the rays and vessels, while the unpitted ones cannot partake in this exchange, though, by reason of their greater length and their highly pitted tangential walls, they may be active in the radial transmission of solutes (Plate 1, Figs. 2, 3, 5, 6, and 7).

It has been found that, in *Eucalyptus regnans*, although there is homogeneity of cell shape throughout the ray, certain cells always take up staining reagents differently from the rest, pointing either to a difference in the cell contents, or to a difference in the composition of the cell wall itself. Further examination has shown that this variation in staining corresponds to a difference in function, for, where the ray is in contact with a vessel, the more heavily staining cells are unpitted, and similar differences between the files of cells are found to those observed in *Salix*, etc., though the proportions of the parts is not the same (Figs. 1C and 3B).

This difference in pitting between the marginal and peripheral cells of the ray has been observed in many woods from a variety of families, but the differences in staining reactions noticed in *E. regnans* are by no means universal. This may, however, be due to the majority of the observations having been made on dry material. (Plate 1, Figs. 1 and 4.)

The whole question of differences in cell contents between ray cells—to which reference will be made later—is one that should be thoroughly investigated, as it is now certain that it is the ray cells that play the most important part in the change from sapwood to heartwood.

An intermediate condition between the fully pitted and the unpitted ray cell can be found among the woods of the Sapotaceae, which are characterized by having different types of pitting in erect and procumbent cells. The most usual condition is that shown in Figure 3C (*Palaquium galatoxylum* (F.v.M.) H.J.Lam.), in which the procumbent cells communicate with the vessels

through pits of the same size and shape as the intervessel pits, but those from the erect ray cells to the vessels are larger, elongated, and often irregular, appearing to result from the fusion of several pit fields into one pit during differentiation. A variant on this condition appears in the uniseriate rays of *Madhuca utilis* H.J.Lam., in which there is a marked correlation between erect ray cells and large pits, though the distribution of the different types of cell through the ray is somewhat irregular (Fig. 4A). Two species of *Chrysophyllum*, *C. pruniferum* F.v.M., and *C. roxburghii* G.Don. (Fig. 4B,C), illustrate a further stage of specialization, the erect cells showing a mixture of pit sizes and shapes, while the procumbent cells are pitted very sparsely, if at all, with pits similar in size and shape to the intervessel pits.

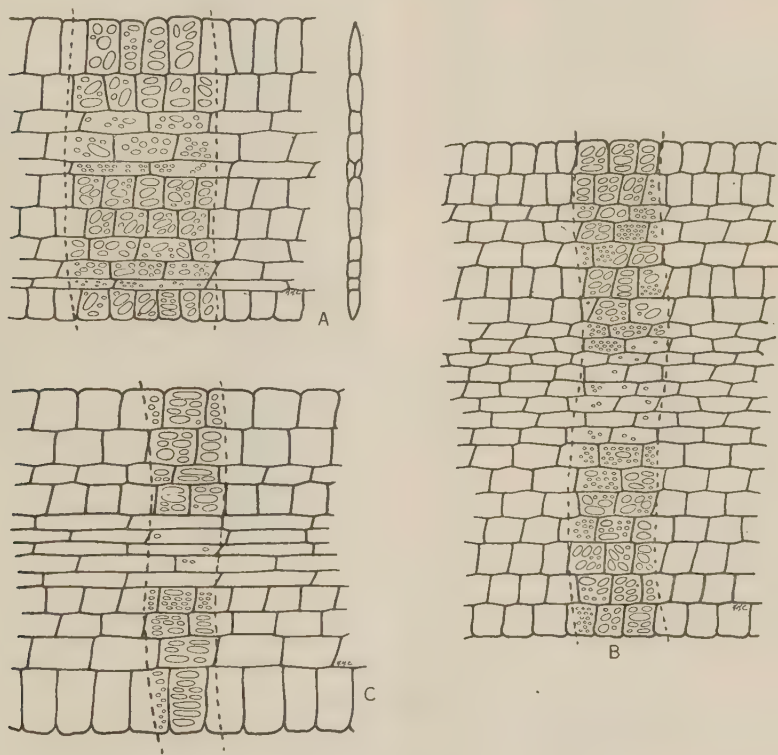


Fig. 4

A, *Madhuca utilis* H.J.Lam. (Sapotaceae).

B, *Chrysophyllum pruniferum* F.v.M. (Sapotaceae).

C, *Chrysophyllum roxburghii* G.Don. (Sapotaceae).

(All $\times 110$.)

Differences in pitting between different files of ray cells are not confined to any particular type of ray; they have been found in uniseriate rays, both those with (*Salix*, *Populus*) and those without erect marginal cells (*Eucalyptus*), and in multiseriate rays (*Acer pseudoplatanus*). But pits are never absent from erect cells, either in uniseriate rays or multiseriate ones, though their absence

from the procumbent cells is common and spread through many different families. As phylogenetic studies have shown that specialization tends towards the elimination of erect cells and the establishment of the homogeneous ray, the absence of pitting may be regarded as another sign of specialization in the allocation of different functions to different parts of the ray.

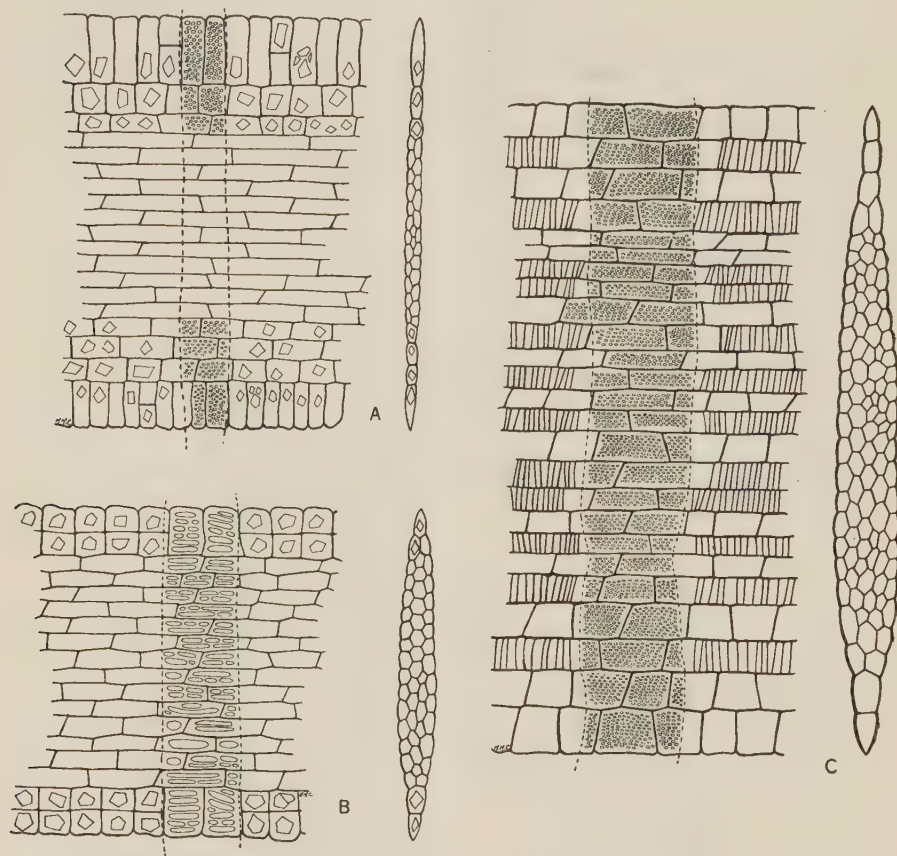


Fig. 5

A, *Cyclostemon* sp. (Euphorbiaceae).

B, *Canarium mehenbethene* Gaertn. (Burseraceae).

C, *Durio oxleyanus* Griff. (Bombacaceae).

(All $\times 110$.)

(c) Cell Contents

Crystals are usually found in the wood rays and wood parenchyma, more rarely in the fibres (Chattaway 1937, 1939). Within the ray they may occur in either erect or procumbent cells, or in cells similar in shape to other ray cells or specially enlarged or subdivided.

It has been observed that, in rays with crystalliferous cells, the cells contiguous with the vessels do not contain crystals, and this is often mirrored in their shape. Figure 5A shows that in *Cyclostemon* sp. both crystals and ray-vessel

pits are confined to the marginal cells, but that crystals are absent from the pitted cells. The cells of the unpitted files are procumbent. A similar distribution of crystals is shown in *Canarium mehenbethene* Gaertn. (Fig. 5B), but the peripheral procumbent cells are pitted with similar pitting to the erect cells. In this wood the crystalliferous cells appear to have arisen through extra-cambial divisions of a single file of marginal cells. These extra-cambial divisions do not occur when the cells are contiguous with a vessel.

Hopea nutans Ridl. illustrates another variant (Fig. 3A). All the ray cells are pitted when they are contiguous with vessels, and files of short, almost square, crystalliferous cells are interspersed throughout the ray. Here too the cells of the crystalliferous files are without crystals when they are in contact with the vessels.

The importance of crystals in the metabolism of the tree is not yet fully understood. They are probably by-products of the differentiating tissues, for they appear in the wood immediately upon its differentiation from the cambium. The cells in which they occur retain their nuclei and take part in the normal metabolism of the tree. Starch may be formed in and resorbed from crystalliferous cells in exactly the same way as from other cells of the rays or parenchyma.

In some woods the distribution of dense cell contents through the rays appears to be correlated with the shape of the cells. This is most clearly illustrated in rays with *tile cells*, a specialization found in certain genera of the Malvales (Chattaway 1933a). *Durio oxleyanus* Griff. is illustrated in Figure 5C and Plate 2, Figure 3, and *Boschia griffithii* Mast. in Plate 2, Figures 1, 2, and 4. The development of these specialized rays will be discussed in detail later, in the section on ray development.

In *Acacia*, *Eucalyptus*, *Nothofagus*, and many other genera, the rays contain both starch and tannins, which occur together in all the ray cells. In *Argyrodendron*, on the other hand, there is a segregation of starch and tannin, the starch occurring in the vertical parenchyma and the erect ray cells, while the tannins are confined to the procumbent cells. The distribution of cell contents must, therefore, undergo a change as the ray grows from a uniseriate ray, composed of starch-containing erect cells only, to the multiseriate condition in which the erect, starch-bearing cells are marginal or peripheral (sheath cells), while the central procumbent cells of the ray contain tannin. The significance of these changes will be discussed in the following section.

(d) Ray Development

The full importance of all these variations in cell shape, pitting, and contents is not yet understood. The best method of study is to follow the ontogeny of individual rays through the wood. As the perimeter of the stem increases, the existing rays become further apart and new ones arise between them to maintain the even distribution of this vital tissue through the tree trunk. The new rays are at first uniseriate, and are, in the majority of woods, formed entirely of erect cells. In the course of growth and development some of these

rays divide to form a multiseriate portion, the cells of which may retain their erect form, as in *Pipturus argenteus* (Fig. 1B), or change from erect to procumbent.

The majority of the changes involving growth that take place in the wood occur at the cambium, either as a result of changes in the initial itself or in the daughter cells very soon after these have been cut off from the cambial initial. Secondary thickening fixes these changes in cell shape, and once secondary thickening of the cell walls has taken place their plasticity is lost and the rigid nature of the wood prevents further marked changes of shape or size. The exception to this occurs when the unthickened pit membrane of a ray cell is pushed through the pit mouth to form a tylosis. This occurs as the result of some special stimulation, such as injury, usually towards the end of the life of the ray cell (Chattaway 1949). Apart from this renewed burst of growth, the activities of the living ray cells appear to be confined to the metabolic processes of radial conduction and storage.

During periods of active growth throughout the tree, the ray initials in the cambium may be dividing by tangential walls to keep pace with the increasing radial diameter of the stem, and by radial and horizontal walls to increase the size of the ray tangentially and vertically. In rays such as those of *Pipturus argenteus*, which are found among the less highly specialized woods, the cells are all erect and there has been little extra-cambial extension. In such woods the cambial divisions may be assumed to have kept pace with those of the fusiform initials. This stage of phylogenetic development can be found repeated in the ontogeny of many trees, where the rays are found in the region near the pith, to consist of narrow erect cells only, even when the rays of the mature wood show considerable specialization.

Further specialization leads to rays formed of uniseriate margins of erect cells and multiseriate parts formed of procumbent cells. During the radial growth of such rays by cambial divisions the erect margins must grow by cell division while the central cells are extending radially. This implies one of two alternatives at the actual cambial layer itself, either some cells of the group of ray initials are dividing much more rapidly than others, or, as seems more likely, all the initials are dividing at the same rate to give groups of daughter cells; very soon these begin to differ in contents so that some of them swell and undergo extension in the only possible direction — radially — while others divide more rapidly by tangential walls, keeping pace with the radial expansion of the other cells.

Investigations carried out on the rays of certain Malvales (Chattaway 1933b) showed that during the development of tile cells the procumbent cells of the ray were actually extending while extra-cambial divisions were taking place in the daughter initials of the erect cells which, in this case, divided so rapidly that as many as ten or a dozen erect cells were formed while one procumbent cell was extending. In green branch material of *Guazuma tomentosa* H.B. & K., there appeared to be differences in cell contents in the cambium itself, but more recent studies of mature wood of other genera suggest that in

some woods the differences in cell contents can also appear in the daughter cells immediately after they have been cut off by the cambium. It is always very difficult, among the several thin-walled cells of the cambial layer, to distinguish between the cell that remains forever in a meristematic condition as the initial mother cell and the daughter cell that will shortly undergo differentiation and secondary thickening.

Material of *Durio oxleyanus* and *Boschia griffithii* recently examined suggests that the difference in cell contents sometimes originates in the daughter cell and not in the cambial initial itself, as procumbent cells with dense contents may be interspersed in files of the tile cells. In these woods (Fig. 5C and Plate 2, Figs. 1-4) all the files of ray cells are pitted when they are contiguous with the vessels, but the cells of the tile cell rows are, when in contact with a vessel, no different from the cells of the procumbent files. This similarity extends to the cell contents as well as to the shape (Plate 2, Figs. 1-4). It appears that, in this case, it is the contents that determine the shape, for the cells with dark contents may occur among empty tile cells not only around the vessels, but also sporadically distributed throughout the ray. When this occurs (Plate 2, Figs. 1 and 2) the cells are always somewhat elongated radially and never of the characteristic "tile" shape.

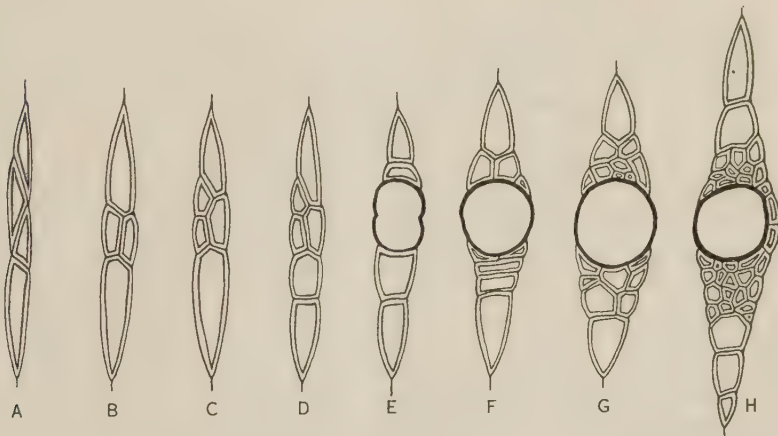


Fig. 6.—*Dyera costulata* Hook. f. (Burseraceae). Serial tangential sections of a ray showing the development of a mucilage canal. The canal has been formed by the breaking down of a wall between two ray cells. x 175.

The segregation of starch and tannin into the erect and procumbent cells is of particular interest when it is considered in connection with the growth and development of the rays. It has been shown (Chattaway 1933b) that, in species of *Argyrodendron*, the erect marginal cells and also the *sheath cells* are often products of a cambial initial that has only recently changed in function from a fusiform initial producing fibres, parenchyma, and vessels, to a column of initials producing ray cells. This change means that horizontal divisions of the initial itself have replaced extra-cambial divisions of the daughter cell, which became a parenchyma strand (Fig. 7A-E).

The subdivided daughter cell of the fusiform initial (the parenchyma strand) and the daughter cells of a uniseriate group of ray initials (the uniseriate ray) are very similar in appearance on tangential sections, and they are almost identical in their contents. However, at a later stage in the growth of the ray, the ray initials subdivide again, to produce a multiseriate ray, and the column of initials that has been added to the side of an existing ray as a row of sheath cells undergoes subdivision into smaller cells. At this stage there appears, not only a difference in size between the two types of cell, but also a difference in contents, and this difference is maintained in the subsequent history of the cells. The marginal and sheath cells undergo tangential division (which may be extra-cambial) but the procumbent cells undergo radial extension.

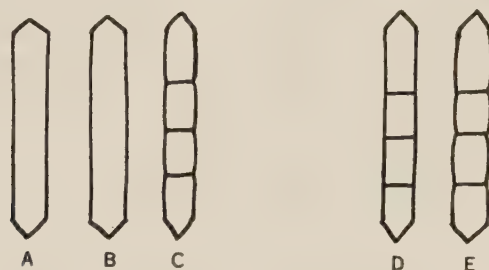


Fig. 7.—Diagrams illustrating the difference between extra-cambial and cambial divisions.

A, Fusiform cambial initial; B, daughter cell of this initial; C, later stage of the same daughter cell after it has undergone extra-cambial divisions which produce a column of four parenchyma cells; D, four ray initials; E, daughter cells of these initials, which form a four-celled uniseriate ray.

The even spread of ray tissue through the increasing circumference of the stem is not always attained by the change from fusiform initials to ray initials, but is also achieved by the breaking up of large rays. When this occurs the process described above is reversed, the radially elongated daughter cells being replaced by others that produce only erect cells. The subdivisions in the cambial initial itself are lost and the initial reverts first to a column closely resembling sheath cells and finally to a fusiform cambial initial from which fibres, vessels, or parenchyma strands are cut off in the normal sequence of the wood (Chattaway 1933*b*, 1937).

During investigations into the structure of the Proteaceae (Chattaway 1948) it was observed that, in *Banksia* and *Dryandra*, certain rays contain vascular tissue which consists of vessels and tracheids traversing the wood horizontally. These vascular strands, which are continued into the phloem, originate through changes in the cambial initials of existing rays, and are developed in connection with the vessels and tracheids of the secondary wood. Their function is obscure, but they seem to be produced in response to a need for the

radial conduction of water. Similar vascular strands have now been observed in *Lagunaria patersonii* G. Don (Malvaceae).

Recently the development of horizontal secretory canals in the rays of both pored and non-pored timbers has been studied by the serial tangential section method (Chattaway 1951). These canals have been shown to arise through changes in the cambial initials that cause a permanent alteration of the type of cell within the ray. The changes in the cambium result, in pored timbers, from some stimulus that causes vertical canals to develop in the secondary phloem.

The laticiferous tubes of the Apocynaceae and the mucilage canals of the Burseraceae appear to develop as the result of some stimulus in the inner phloem, similar to that causing the radial gum canals. Owing to the crushing of the soft tissue of the phloem of the material examined it was not possible to trace their development in the phloem, but their appearance suddenly in the rays of the wood without any obvious vertical connections (Fig. 6A-H) and the absence of any similar structures from the vertical elements of the xylem suggest that, like the secretory canals mentioned above, they originate in connection with similar tissue in the phloem.

III. DISCUSSION

At the commencement of this paper, three main points were set out for elucidation; what differences there are in function between the different cells of a ray, whether these differences are due to the position of the cells in the ray, and how far cell contents may be said to determine the differences in cell shape.

The examples cited above show that the wood ray may be a very complex tissue, the elements of which, even when they differ little from one another in appearance, are functioning in a variety of ways. Little work has been done on many of the functions of ray tissue, the general dispersion radially of manufactured food materials having, in the past, been considered as the most important of its activities.

As can be seen from the foregoing pages, the rays are in fact a tissue system as varied as that of the vertical elements of the wood, and the cells of which they are composed may serve for storage, conduction, or secretion just as do the similar elements of the vertical system. The rays may include such different elements as vessels and tracheids, horizontal secretory canals, laticiferous tubes, cells for the storage of starch, oil, silica, crystals, etc. As in the vertical system, not all of these specialized cells occur in all woods; storage cells are more common than secretory ones, and vascular tissue, which is so prominent a feature of the vertical system, is rarely found in the horizontal. Furthermore, the presence of one type of tissue in the vertical system does not imply that it is also present in the horizontal, or vice versa. Vascular tissue is the most obvious example of this, but further examples can be found in every type of tissue cited. Both crystals and silica may each be present in both ray cells and vertical parenchyma, but in some woods they may occur only in the vertical or only in the radial parenchyma. Similarly, the oil cells of the Lauraceae may

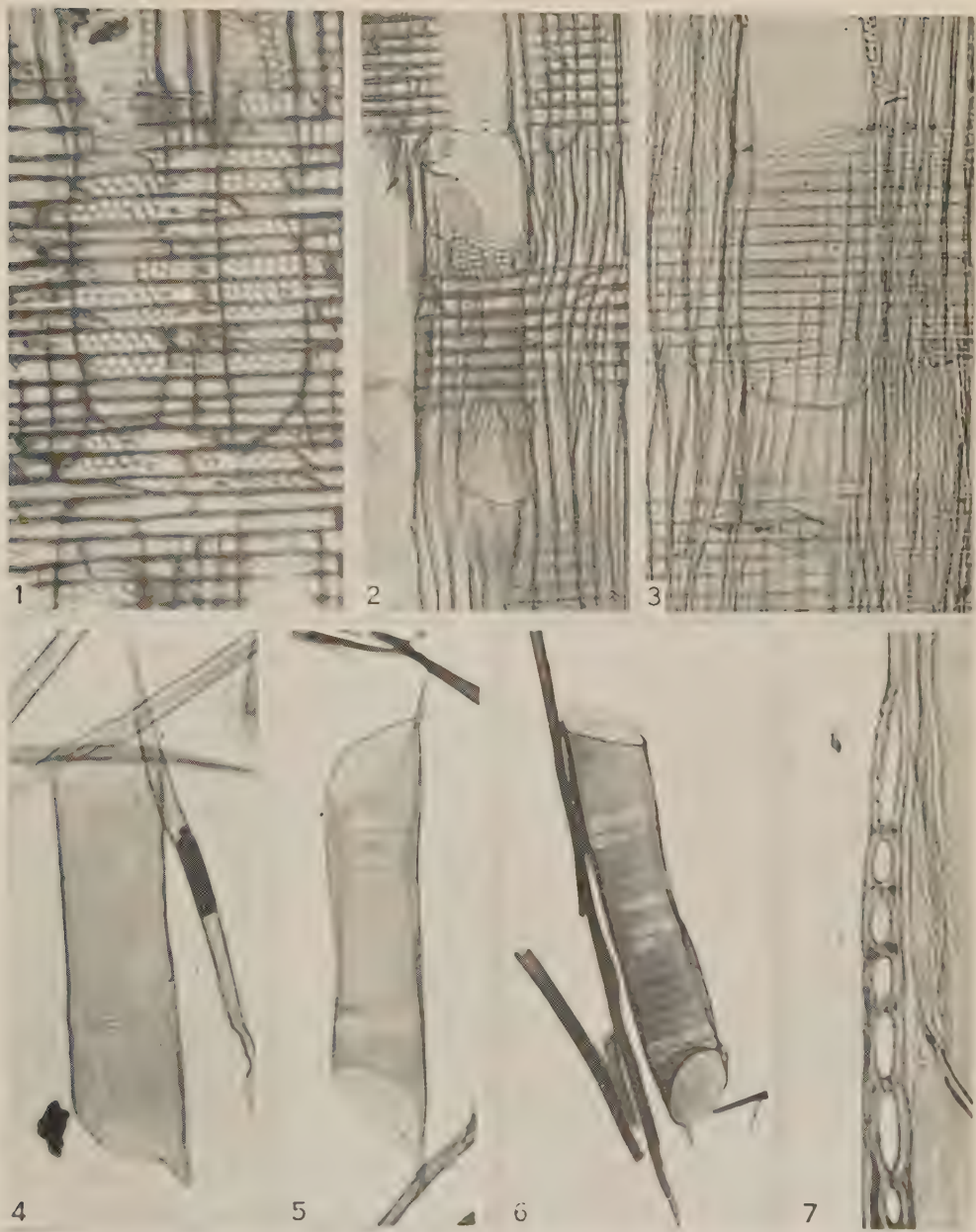
be found in the rays only, in the vertical parenchyma only, or in both these tissues in the same wood. Horizontal secretory canals are found in the rays of genera of the Anacardiaceae, Burseraceae, etc., but are absent from the vertical elements of these woods; in the Dipterocarpaceae they are a regular feature of the vertical tissue of the wood of many genera, but are absent from the rays except in a small subsection of the genus *Shorea*.

As all the cells of both the vertical and horizontal systems originate in the cambial layer, it is there that the changes must be looked for as one type of cell gives place to another. In the vertical system all the cambial initials are similar at the time of their formation, though they may be differentiated very early into the different elements of the wood (vessels, fibres, parenchyma strands, etc.) or of the phloem (sieve tubes, companion cells, phloem fibres, etc.). If the elements finally differentiated are subdivided horizontally the divisions must have been extra-cambial, as the same initial continues to produce a radial sequence of undivided fusiform daughter cells (Fig. 7A-E). Sometimes, however, the series of divisions may occur in the initial itself, taking a permanent place in the wood and producing cells shorter vertically and in a continuous radial series (Fig. 7D,E). The fusiform initial has in this case changed into a column of ray initials. When such a uniseriate ray becomes multi-seriate the divisions again occur in the initials themselves and are mirrored in all the cell series subsequently cut off by them. This is a very important distinction between the ray and the fusiform initials, for it means that whereas the divisions of the fusiform initial cut off tissue that becomes wood on one side and phloem on the other, the ray initials produce ray tissue on both sides of the cambial layer, thus preserving the continuity of radial conduction from the phloem into the xylem.

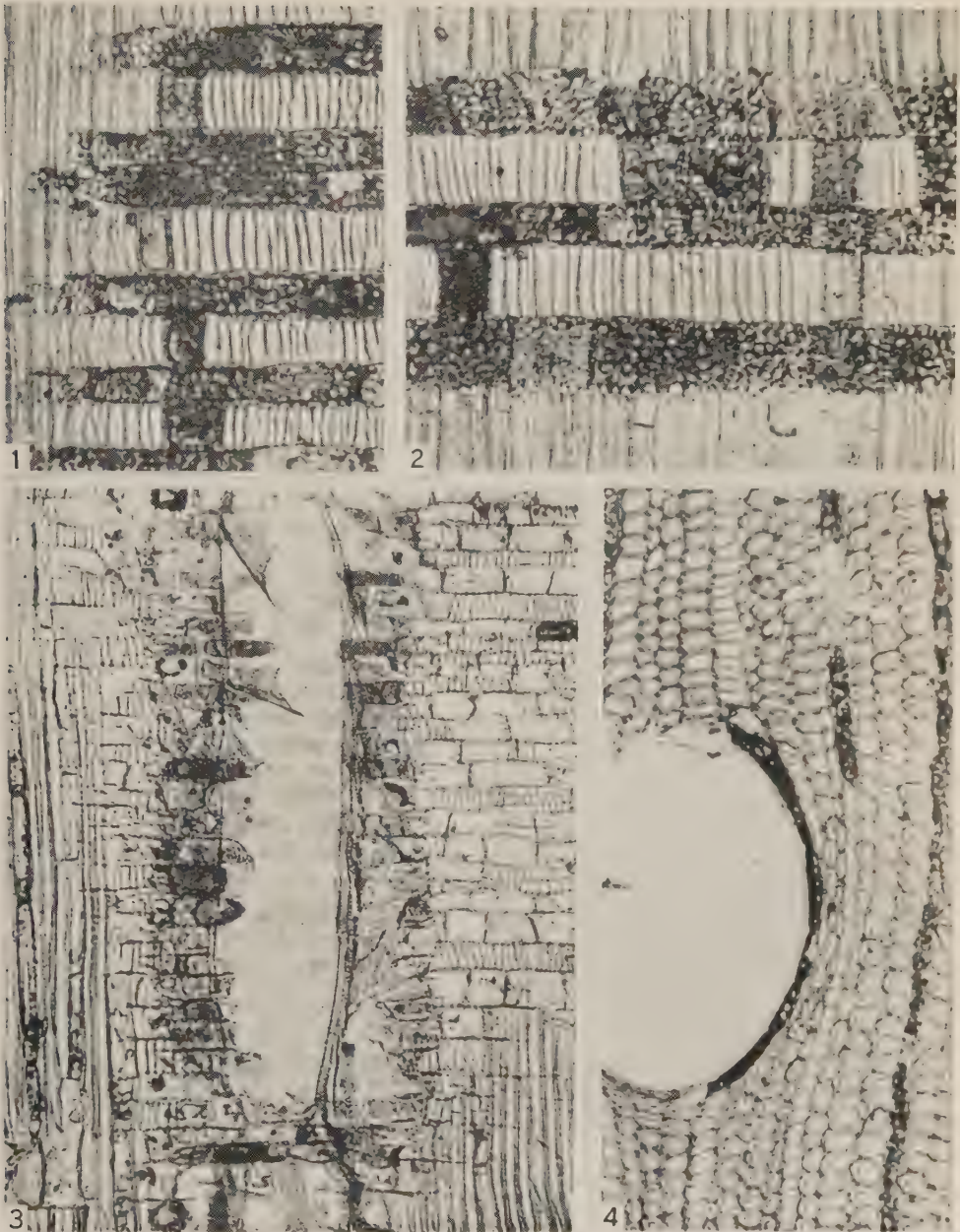
As the vessels, fibres, and parenchyma of the vertical tissues result from extra-cambial changes, so also do many of the cell variations that are found in the rays; these can be distinguished by their discontinuity in the radial series. For example, in *Cyclostemon* sp. (Fig. 5A), the horizontal divisions that produced the inner cells, which are shorter vertically than the marginal ones, were in the cambial initials, for these cells continue at relatively the same height for a long distance through the ray. But the marginal cells in which the crystals lie are occasionally only half the normal height, having undergone extra-cambial division, which has occurred in one daughter cell but not in the next. A more extreme example is seen in *Canarium mehenbethene* (Fig. 5B). Here the extra-cambial divisions that give rise to the crystal cells have been more numerous and form a fairly continuous radial series, but they are interrupted where the ray is contiguous with a vessel. These cells are the full height of the initial; they are not subdivided and they do not contain crystals.

These two woods are sufficient to illustrate the difficulty of interpreting the influence of cell contents on cell shape. In *Cyclostemon* sp. the crystals are usually present in the unaltered ray cells; in *Canarium mehenbethene* the cells that contain them appear to have been subdivided so that a single crystal fills each cell.

VARIATIONS IN RAYS OF PORED TIMBERS



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The influence of cell contents on pitting has been equally difficult to assess, as most of the work has been done on dry material from the collection of the Division of Forest Products. Even when fresh material has been available the evidence is conflicting. In *Eucalyptus regnans* the files of pitted cells appear to differ in staining properties from those of the unpitted cells, but no such differences could be observed in *Nothofagus cunninghamii* though the same distinction between pitted and unpitted files occurs. That differences in osmotic pressure occur has been shown by Kny (1909), but it would appear that these result from the differences in pitting and not that they are responsible for them. The highest osmotic values were found in the unpitted files, and the least in the pitted marginal cells, while the unpitted marginal cells (which are not contiguous with the vessels) were intermediate. It is possible that the absence of crystals from cells contiguous with the vessels may be due to the removal in the sap stream of certain of the by-products of metabolism, but in such a wood as *Cyclostemon* sp. another explanation must be sought for their absence from the cells of the unpitted files, and also for their absence from the procumbent files of cells in *Canarium mehenbethene*, in which the pitting to the vessels is similar in the erect and procumbent files. The whole question of cell inclusions is clearly a most complex one, calling for special microchemical technique and for freshly felled green material of the cambial regions of mature trees, for both crystals and silica appear to be formed in the cells as soon as these are differentiated from the meristematic layer.

In the foregoing pages an attempt has been made to show how the different radial tissue systems develop to keep pace with the increasing girth of the tree, and in the course of this study the interdependence of the radial and vertical systems at the cambial layer has become very clear. Other investigations (Chattaway 1949, 1951) have shown that the vascular tissue and secretory passages in the rays develop only in connection with the vertical tissues. The former originate in connection with the vertical conducting tissue of the wood and the latter in the canals of the phloem or (in the Coniferae) of the xylem. These are both relatively uncommon features, developed only in a few woods, and may be considered to be lines of specialization that are somewhat apart from the main phylogenetic sequence of ray development. The sequence from rays with high erect margins to rays that are composed of procumbent cells only has been shown to run parallel with other sequences of specialization, such as the replacement of scalariform by simple perforation plates. However, the same sequence of ray development is often found in one tree in the wood developed from the centre to the periphery, or from the initiation of a newly formed uniseriate to the multiseriate condition. This sequence must have its origin in actual changes in the shape and development of the cambial initials.

Changes in cell contents as one type of cell file gives place to another must arise through changes in the metabolism at the cambial layer. That one cell file of the ray may be dividing while another is expanding radially is understandable if the cell contents differ, or if the surrounding cells of the differentiating layer produce differences in metabolism. Such differences may explain

the greater radial length of central ray cells as compared with the peripheral ones, and the differences between cells contiguous with vessels and those that are not. But the cause of erect ray initials subdividing by radial and horizontal walls while they maintain the same position in the ray in relation to the other elements, and at the same time producing daughter cells that elongate radially instead of undergoing tangential division, implies a fundamental change in the metabolism of the initial itself. Such a change occurs in many woods when a uniseriate ray composed of erect cells becomes partially biseriate.

The work on secretory canals has shown that changes in the ray initials may owe their origin to influences either in the wood or in the phloem. Much recent work has concentrated on the differentiation of the wood because it is the wood that is of primary commercial importance today. But the problem is not one of the wood alone, especially where ray tissue is concerned, and the answers to problems of ray development in the wood may have to be looked for in the phloem. The vertical elements of wood and phloem develop independently on either side of the cambial layer.* In the past, ray structure has been considered mainly from its appearance in the wood and the general metabolism of the different types of cell included in the rays has been ignored. It is accepted, however, that many of the materials stored in the ray cells have entered from the phloem, so that the origin of the cells in which they are stored might logically be found also in that part of the tree. At present the metabolism of the different ray cells is little understood, though work in progress at the Division of Forest Products on the sapwood-heartwood change and on siliceous timbers is showing how fundamental it is to the elucidation of many problems connected with timber. It may well be that a study of the phloem will provide an answer to some of these questions that has not been found through studies of the wood alone.

IV. ACKNOWLEDGMENT

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V. REFERENCES

- BARGHOORN, E. S. (1940).—*Amer. J. Bot.* **27** (10): 918-28.
 CHALK, L., and CHATTAWAY, M. M. (1937).—*Trop. Woods* **50**: 1-31.
 CHATTAWAY, M. M. (1933a).—*New Phytol* **32** (4): 261-73.
 CHATTAWAY, M. M. (1933b).—*Forestry* **7** (2): 93-108.
 CHATTAWAY, M. M. (1937).—*Philos. Trans. B.* **554** (228): 313-66.
 CHATTAWAY, M. M. (1939).—*Roderiguesia* **12**: 55-8.
 CHATTAWAY, M. M. (1948).—*J. Coun. Sci. Industr. Res. Aust.* **21** (4): 275-8.
 CHATTAWAY, M. M. (1949).—*Aust. J. Sci. Res. B* **2** (3): 227-40.
 CHATTAWAY, M. M. (1951).—*Aust. J. Sci. Res. B* **4**: 1.
 INTERNATIONAL ASSOCIATION OF WOOD ANATOMISTS (1933).—*Trop. Woods* **36**: 1-12.
 KNY, L. (1909).—*Landw. Jb.* **38**: 375-94.

* It is only rarely that internal phloem develops within the woody cylinder (Chalk and Chattaway 1937). The woods in which it occurs belong to only a few families and its occurrence is often associated with other indications of a high degree of specialization.

KRIBS, D. (1935).—*Bot. Gaz.* **96** (3): 547-57.

MOLL, J. W., and JANSSONIUS, H. H. (1906-36).—*Mikrographie des Holzes der auf Java Vorkommenden Baumarten.* (Brill: Leiden.)

REINDERS-GOUWENTAK, C. A. (1949).—*Meded. LandbHoogesch. Wageningen* **49**: 217-36.

EXPLANATION OF PLATES 1 AND 2

PLATE 1

Fig. 1.—*Acer pseudoplatanus* L. Radial longitudinal section showing pitted and unpitted ray cells interspersed throughout the ray. x225.

Fig. 2.—*Salix caerulea* Sm. Radial longitudinal section showing pitted ray cells confined to the marginal rows. x125.

Fig. 3.—*Populus serotina* Hartig. As for Figure 2, but with more than one row of pitted cells. x125.

Fig. 4.—*Acer pseudoplatanus* L. Vessel member isolated by maceration, showing ray-vessel pitting. x125.

Fig. 5.—*Salix caerulea* Sm. Vessel member isolated by maceration, showing ray-vessel pitting. x120.

Fig. 6.—*Populus serotina* Hartig. Vessel member isolated by maceration, showing ray-vessel pitting. x100.

Fig. 7.—*Salix caerulea* Sm. Tangential longitudinal section showing pitted and unpitted ray cells. x400.

PLATE 2

Fig. 1.—*Boschia griffithii* Mast. Radial longitudinal section showing tile cells without contents and radially elongated ray cells with dense contents. Cells with contents, which occur among the tile cells, are always longer radially than the accompanying tile cells. x185.

Fig. 2.—As for Figure 1.

Fig. 3.—*Durio oxleyanus* Griff. Radial longitudinal section showing ray-vessel pitting. All the cells are radially elongated when they are in contact with a vessel. x110.

Fig. 4.—*Boschia griffithii* Mast. Transverse section showing tile cells replaced by radially elongated cells with dark contents where the ray is contiguous to a vessel. x185.

RADIO-AUTOGRAPHS OF MANGANESE IN PLANTS

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Summary

Flax, peas, cabbage, and tomato plants were grown in nutrient solutions to which radio-active manganese (0.035 mc. of Mn^{54}) was added with an excess of non-labelled manganese. The treatments included an excess of molybdenum as ammonium molybdate or sodium molybdate in addition to the excess manganese. With flax and peas a further radio-active treatment was studied in which the total manganese level was normal (0.5 p.p.m.). Radio-autographs and radio-assays of various plant tissues were made.

The oldest leaves of the plant were higher in manganese than the youngest leaves. There was a characteristic accumulation of manganese in the distal third of flax leaves, in the margins of leaflets and stipules and the tips of tendrils of peas, in the margins of cabbage leaves, and in the terminal leaflet and tips of other leaflets of tomato leaves. With flax and peas the above accumulations were demonstrated irrespective of whether there was a normal or excess level of manganese in the nutrient solution. Before the onset of necrosis it was also shown with peas, cabbage, and tomato that the accumulation of manganese occurred in the interveinal tissues.

Flax plants receiving the normal manganese level showed, in addition to the leaf tip accumulation, numerous minute localized concentrations of manganese throughout the tissues of the older leaves and cotyledons, and, ultimately, the base of the stem. This phenomenon occurred to a very limited extent only at the excess manganese level.

In the presence of applied molybdenum the plants in general had a lower concentration and a more uniform distribution of manganese. In certain instances the distribution was quite abnormal in that the leaf centre had a higher concentration of manganese than the corresponding leaf edge.

The onset of necrosis was associated with a marked accumulation of manganese in the tissues concerned. However, it was further demonstrated with peas, cabbage, and tomato that there is actually a withdrawal of manganese from the tissues as the necrosis becomes complete.

Successive radio-autographs of the same pea plants have shown that a movement of manganese from the interveinal tissues into the veins occurs as the plant dries out. This indicates the possibility that radio-autographs may not provide a true pattern of the location of the manganese as it occurs in the living plant.

I. INTRODUCTION

It has been shown (Millikan 1947, 1949, 1950) that the effects on flax of manganese in the nutrient solution may be profoundly affected by several factors, including nitrogen source and molybdenum supply. In continuation of this work, the present experiments were designed to determine, by the use of radio-active manganese (Mn^{54}), the distribution of manganese in plants grown

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in solutions containing a normal or excess level of this element. The effect of excess molybdenum, applied in conjunction with an excess of manganese, was also studied.

The radio-autograph technique was primarily used to provide this information. However, radio-assays were also made to confirm the existence of differences in manganese concentration in various tissues of the plants as revealed by the radio-autographs.

II. METHOD

The general method employed for the conduct of nutrient solution cultures was similar to that described previously (Millikan 1950). All experiments were performed out of doors. The pH of each solution was adjusted to 4.5 twice a week. However, to avoid contamination of the pH meter and other equipment, the reactions of the solutions containing radio-active manganese were adjusted to pH 4.5 on the basis of determinations made on a series of non-radio-active control cultures containing identical amounts of manganese and other chemicals. All treatments were set up in duplicate.

The total concentrations of manganese added to the various cultures were: flax, 50 p.p.m.; peas, cabbage, and tomato, 25 p.p.m. Further radio-active treatments containing a total of 0.5 p.p.m. of manganese were included in experiments with peas and flax. In the radio-active series, each culture received 0.035 mc. of Mn^{54} . This isotope has a half life of 310 days. An excess of molybdenum (20 p.p.m.) as either ammonium molybdate or sodium molybdate was added to certain cultures in conjunction with excess manganese.

The number of plants per culture was: flax, ten; peas, six; cabbage and tomato, five, respectively. The flax and pea seed was germinated in washed sterilized sand, and the seedlings were transferred to the cultures approximately two days after emergence. The cabbage and tomato seedlings were grown in soil in the greenhouse and were transferred to the cultures about two to three weeks after emergence. At appropriate times samples of plants were taken either for the preparation of radio-autographs, or for radio-assays for manganese.

In making gross radio-autographs, Kodirex no-screen X-ray film was used. The whole plant or part, as the case may be, was first pressed flat and then placed on the film in the dark room. Where the plant material was fresh, it was found desirable to place a thin piece of cellophane between the film and the specimen, to prevent damage to the emulsion. The film and specimen were next mounted between two glass plates, which were pressed tightly together and then bound firmly with "Durex" tape. The exposure time varied from approximately 6 to 12 days, according to the specimen. During this time the films were stored in light-tight boxes in a refrigerator. In opening up the glass plates to get the film after exposure, it was found essential that the "Durex" tape be cut along the edges of the glass plates, and not pulled off the glass in any way, as a flash of light sufficient to fog the film is produced as the tape leaves a glass surface.

For the radio-assays, composite samples were obtained from the duplicates of each treatment. The plants concerned were cut up into the required parts. Where the leaf or stipule edge and centre were assayed separately, the edge consisted of a strip approximately 3 mm. wide, and the remaining portion was designated the centre. With flax, the leaf top consisted of the distal third, and the leaf base the remaining two-thirds of the leaf. Similarly with pea tendrils, the top consisted of the distal third, and the base the remaining two-thirds. Each sample was placed in a small aluminium pan appropriately labelled beneath with a grease pencil, and was dried in an oven at 105°C. for at least three hours, after which the dry weight was obtained to the nearest 0.0001 g. The sample was then finely ground, spread evenly over the aluminium pan, and placed in a standard lead castle fitted with an EHM2 geiger tube for counting, as described by Oddie and Mibus (1949). The method of calculation of the manganese content of the sample was according to that of Oddie (unpublished data 1950). Manganese determinations made by the radio-assay method showed good agreement with similar determinations made by the conventional ash analysis.

III. RESULTS

(a) *Flax*

The characteristic symptoms of manganese toxicity in flax have been described (Millikan 1949). Briefly, they consist of dwarfing, iron deficiency chlorosis, followed by necrosis of the top of the plant and necrosis of the lower (middle) leaves, commencing at the distal ends.

(i) *Radio-autographs*.—The radio-autographs of plants from the flax experiment reveal a difference in the distribution of manganese in the plant when this element is supplied at the normal (0.5 p.p.m.) and excess (50 p.p.m.) levels respectively. A marked concentration of manganese occurred in the tops of the leaves at both manganese levels (Plate 1, Figs. 2-4). At the normal level a marked accumulation of manganese in small "islands" distributed irregularly over the cotyledons and lower leaves occurred. There were no external symptoms in the leaves that produced these radio-autographs.

Comparing the radio-autographs of the eight-day, 14-day, and 23-day-old plants respectively, it is evident that the occurrence of these localized accumulations of manganese is connected in some way with the age of the tissues (Plate 1, Figs. 2-4). The youngest leaves show no evidence of them, and it is only in the radio-autograph of the 23-day-old plants that they appear for the first time at the base of the stem.

At the excess manganese level, this localization of the manganese is much less apparent. A limited number of "spots" occur in the cotyledons, but not in the leaves, of the eight-day samples. No increase in "spotting" occurred in the 14-day samples, but at 23 days there was first evidence of "spotting" in the lower leaves and the base of the stem. No information is available regarding the particular tissues in which the manganese localization occurs.

In view of the fact that the same amount of radio-active manganese (0.035 mc.) was added to each culture, the proportion of Mn^{54} to the total amount of manganese present in the culture was greater where the total manganese added was 0.5 p.p.m. than where 50 p.p.m. was added. This difference in the relative radio-activity of the manganese doses was reflected in the plants. Using the EHM2 geiger tube, the mean counts per minute per mg. of dry matter for flax plants from the two manganese levels were:

Level of Mn	9-Day Sample	22-Day Sample
0.5 p.p.m.	67.4	109.9
50 p.p.m.	28.5	39.4

Thus, it is not known whether the much greater prevalence of "spotting" in the radio-autographs of the normal flax plants, as compared with those receiving excess manganese, was due to the greater radio-activity of the tissues of the former plants, which would allow any localized manganese accumulations to be more readily recorded in the radio-autographs. The other alternative is that the excess supply of manganese disorganized the normal distribution of this element in the plant.

Radio-autographs of leaves showing lower leaf necrosis, which commences at or near the ultimate tip of the leaf, show that this symptom is associated with a marked accumulation of manganese in the necrotic areas (Plate 1, Fig. 1).

(ii) *Radio-assays*.—The results of radio-assays made on plants from the flax experiment are presented in Table 1.

These results confirm the radio-autographs. An accumulation of manganese in the top third of each leaf occurred, irrespective of whether the plants were grown in cultures containing the normal (0.5 p.p.m.) or excess (50 p.p.m.) levels of manganese, but without an excess of molybdenum.

Where excesses of manganese and molybdenum were added jointly, the plants developed within a few days a characteristic golden-yellow interaction chlorosis of all the leaves, but most prominently of the tips of the older leaves. This manganese-molybdenum interaction has been described (Millikan 1950). The presence of excess molybdenum had a marked effect on the manganese distribution in the leaves. In some instances the normal distribution was reversed in that the leaf base contained a higher concentration of manganese than the leaf top.

(b) *Peas*

The first symptoms of manganese toxicity in field peas consist of a necrosis which appears along the edges of the third or fourth leaflets and corresponding stipules in the form of small greyish spots in the interveinal tissues (Plate 3, Figs. 3 and 4). These marginal spots soon coalesce, and the necrotic edge may inroll. The tendrils of the affected leaves may also show necrosis at the tip. The upper leaves of the plant may show symptoms of iron deficiency chlorosis followed by necrosis.

(i) *Radio-autographs*.—The pea radio-autographs consisted either of individual leaves and stipules, with or without marginal manganese toxicity symptoms, or of whole plants. In the latter case, immediately after the first radio-autograph was obtained, each plant was placed on fresh X-ray film and a second radio-autograph was made.

TABLE 1

FLAX EXPERIMENT. RESULTS OF RADIO-ASSAYS FOR MANGANESE (P.P.M. DRY BASIS), AND SYMPTOMS OF PLANTS SAMPLED AFTER NINE DAYS IN CULTURE

Plant Part *		Mn 50	Mn 50 p.p.m.	Mn 50 p.p.m.	Mn 0.5 p.p.m.
		p.p.m.	Mo (NH ₄)† 20 p.p.m.	Mo (Na)† 20 p.p.m.	
Leaf 2	{ Top	6000	3500	2790	53
	{ Base	3320	2200	3740	45
Leaf 4	{ Top	5550	2670	4080	44
	{ Base	2920	2670	4550	35
Leaf 6	{ Top	7050	4200	3180	56
	{ Base	5660	3260	4270	46
Leaf 8	{ Top	6800	3730	5140	76
	{ Base	4100	3280	3310	56
Cotyledons		1620	1450	1790	41
Stem	{ Top	2230	1610	2350	78
	{ Base	2060	1430	2270	40
Growing Point		4590	2680	4600	95
Symptoms‡		C++ N++	G+++	G+++	— §

* The leaves are numbered from the base of the plant.

† Mo(NH₄) = (NH₄)₆Mo₇O₂₄·4H₂O. Mo(Na) = Na₂MoO₄·2H₂O.

‡ C = iron deficiency chlorosis. G = golden-yellow chlorosis affecting all leaves, but most apparent in the tips of the older leaves. N = necrosis of the older (middle) leaves commencing at the tips. Severity of symptoms: — no symptoms, + very slight, ++ slight, +++ moderate.

§ General growth of these plants was less than the control without Mn⁵⁴.

A marked marginal accumulation of manganese in leaf, stipule, and the tip of the tendril at both normal and excess levels (the latter without added molybdenum) is demonstrated by the radio-autographs. The youngest leaves and the growing point are also shown to be much lower in manganese than the older leaves (Plate 2, Figs. 1 and 2). It has also been demonstrated that the manganese accumulates in the interveinal tissues when supplied to the plant at either the normal or excess level.

Unlike flax, the radio-autographs of pea plants supplied with the normal manganese level have revealed only slight evidence of the accumulation of manganese in small "islands" in the oldest leaves.

In general, the petioles are not high in manganese, but the radio-autographs have shown them to be traversed along their length by one to three highly radio-active, thin lines — presumably corresponding to vascular elements.

Radio-autographs of plants receiving combined excesses of manganese and molybdenum have revealed a much more uniform distribution of manganese between all tissues of the plant than where excess manganese is supplied alone (Plate 2, Fig. 3). This applies particularly to the difference in manganese concentration between leaf or stipule edge and centre, and youngest leaves and growing point, as compared with the older leaves of the plants. There was also a more comparable manganese concentration in stems, petioles, and leaves.

An important phenomenon has been revealed with respect to the development of manganese-induced marginal leaf necrosis. The radio-autographs (Plate 3, Figs. 3 and 4) show that a withdrawal of the manganese from the interveinal necrotic areas, back into the leaf veins, occurs as the necrosis becomes complete.

Reference is made above to the making of two successive radio-autographs of the same tissues. Comparisons of these pairs of radio-autographs have consistently revealed that the location of the manganese in a leaf after the plant is harvested is not fixed. In actual fact, a marked migration of manganese from the interveinal tissues of both leaves and stipules back into the veins has been demonstrated (Plate 4, Figs. 1 and 2). This migration of manganese parallels that from the necrotic areas of the living plant. The plants used to obtain these pairs of radio-autographs were kept closely pressed against the film between two sheets of glass during exposure, so that evaporation from the leaf surface would be very restricted. Whether this increased the movement of sap into the veins is not known.

(ii) *Radio-assays*.—Typical results of radio-assays of pea plants are presented in Tables 2 and 3. In general, they confirm the results of the radio-autographs. Manganese concentration was found to be lower in the youngest leaves and growing point than in the older leaves of the plants. This applies also to the young and old tissues respectively of the basal shoots.

In the absence of excess molybdenum, there was, at both the normal and excess levels of manganese, a marked accumulation of this element in the edges of both leaves and stipules, and in the distal third of the tendril.

The addition of excess molybdenum caused a reduction in manganese concentration in the various tissues of the plant. In addition, the relative difference between edge and respective centre of leaf or stipule, and tendril top and base, was much reduced when compared with corresponding tissues of plants receiving excess manganese without added molybdenum. In some instances the distribution of the manganese in the presence of applied molybdenum was quite abnormal in that the leaf centre contained a higher concentration than the corresponding leaf edge. The smoothing effect of molybdenum on the manganese concentration of the plants confirms the results of the pea radio-autographs.

As regards the occurrence of manganese toxicity symptoms, it should be noted from the results in Table 2 that their severity cannot be related to the

manganese concentration in the tissues. A very high manganese concentration in leaf 1 of the control plants did not cause the development of necrotic symptoms, whereas a much smaller marginal concentration in leaf 5 was associated with necrosis. There may be a fundamental difference between leaf 1 and the younger leaves of the pea plant with respect to manganese tolerance, as the author has rarely observed manganese toxicity symptoms in the former leaf in several unpublished water-culture experiments where excess manganese was studied.

TABLE 2
RESULTS OF RADIO-ASSAYS FOR MANGANESE (P.P.M. DRY BASIS) OF PEA PLANTS
SAMPLED AFTER 15 DAYS IN CULTURE

Plant Part *	Mn 25 p.p.m.	Mn 25 p.p.m. Mo(NH ₄)† 20 p.p.m.	Mn 25 p.p.m. Mo(Na)† 20 p.p.m.	Mn 0.5 p.p.m.
<i>Leaf 1</i>				
Stipule edge	2660	1310	1780	58
Stipule centre	2190	1210	1950	49
Petiole	2100	1730	1870	44
Leaf edge	3290	1990	2420	113
Leaf centre	2600	1760	2220	53
<i>Leaf 3</i>				
Stipule edge	4090	1580	2650	72
Stipule centre	1950	1410	2040	43
Petiole	1450	1070	1290	33
Leaf edge	3160	1250	2400	79
Leaf centre	2450	1770	1960	42
<i>Leaf 5</i>				
Stipule edge	1310	814	1240	34
Stipule centre	994	815	1180	24
Petiole	551	672	782	17
Leaf edge	1450	863	1320	42
Leaf centre	1130	793	1300	26
<i>Stem</i>				
Top	712	1020	888	23
Base	1830	1050	1090	27
Growing point	922	830	741	29
Symptoms‡	3.4.5 N+++	3.4 N+	4 N+	—

* The leaves are numbered from the base of the plant.

† Mo(NH₄) = (NH₄)₆Mo₇O₂₄·4H₂O. Mo(Na) = Na₂MoO₄·2H₂O.

‡ = Marginal necrosis. — No symptoms, + very slight, ++ slight, +++ moderate.

(c) Cabbage

Manganese toxicity in cabbage is associated with the development of iron deficiency chlorosis of the youngest leaves of the plant. At the same time, the middle leaves develop an interveinal chlorosis most pronounced towards the

edges. Marginal necrosis soon follows, and the leaf may become cup-shaped owing to the continued development of the remainder of the lamina. Necrosis may finally extend towards the centre of the lamina in the interveinal tissue. Necrotic "spotting" of the lamina may also occur. A histological examination has shown that this type of necrosis first appears in the palisade cells immediately below the upper epidermis, but finally extends through the leaf to the lower surface.

TABLE 3
RESULTS OF RADIO-ASSAYS FOR MANGANESE (P.P.M. DRY BASIS) OF TENDRILS OF
PEA PLANTS AFTER 22 DAYS IN CULTURE

Plant Part *	Mn 25 p.p.m.	Mn 0.5 p.p.m.
<i>Leaf 3</i>		
Tendril top	1460	128
Tendril base	219	48
<i>Leaf 4</i>		
Tendril top	1810	35
Tendril base	454	27

* The leaves are numbered from the base of the plant.

(i) *Radio-autographs*.—Individual leaves only were radio-autographed. It was found that the manganese accumulates around the leaf margin. At first the vein endings at the margin become very radio-active, suggesting a guttation effect, or secretion of manganese through the hydathodes (Plate 5, Fig. 1). Manganese accumulates in the interveinal tissues in a diffuse manner (Plate 5, Fig. 2), but later in sharply defined spots (Plate 5, Fig. 3), which correspond with the development of necrotic areas. After the necrotic spot is fully developed, i.e. when it has extended from the upper through to the lower surface of the leaf, the dead tissue becomes low in manganese (Plate 5, Fig. 4).

(ii) *Radio-assays*.—The results of radio-assays made on cabbage plants are presented in Table 4.

The characteristic marginal accumulation of manganese in the cabbage leaves is demonstrated by these figures, irrespective of molybdenum level. The oldest leaves of the plants are also highest in manganese.

Marked differences in the effects of excess molybdenum applied as either ammonium molybdate or sodium molybdate respectively on manganese concentration in the tissues and the severity of manganese toxicity symptoms are indicated in Table 4.

(iii) *Analyses of Field-Grown Brassica Plants*.—In view of the marginal accumulations of manganese in leaf edges of plants demonstrated by the radio-autographs and radio-assays, ash analyses were made to determine whether a similar accumulation was a feature of field-grown *Brassica* plants.

To this end, leaf samples (petioles excluded) were obtained from a commercial crop of brussels sprouts, portion of which showed symptoms of manganese deficiency. The healthy and affected portions of the crop were sampled

separately. A strip approximately $\frac{1}{2}$ in. wide was cut off the edges of the leaves. This was designated the leaf edge, and the remainder the leaf centre.

The results of the ash analyses, on a dry basis, were:

Plant Part	Mn (p.p.m.)	
	Healthy Plants	Affected Plants
Leaf edge	15	10
Leaf centre	11	5

The results indicate that a marginal accumulation of manganese occurs in the leaves of field-grown *Brassica* plants.

TABLE 4

CABBAGE EXPERIMENT. RESULTS OF RADIO-ASSAYS FOR MANGANESE (P.P.M. DRY BASIS), AND PLANT SYMPTOMS AFTER 21 DAYS IN CULTURE

Plant Part *	Mn 25 p.p.m.	Mn 25 p.p.m. Mo(NH ₄)† 20 p.p.m.	Mn 25 p.p.m. Mo(Na)† 20 p.p.m.
<i>Leaves 1 and 2</i>			
Lamina	1800	1010	2780
<i>Leaves 3 and 4</i>			
Lamina edges	2710	1600	3200
Lamina centres	590	370	910
Petioles	320	310	370
<i>Leaves 5 and 6</i>			
Lamina	770	390	810
Symptoms‡	C++ N+++ IC++	—	C++ N++ IC+++

* The leaves are numbered from the base of the plant.

† $\text{Mo}(\text{NH}_4) = (\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$. $\text{Mo}(\text{Na}) = \text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$.

‡ C = iron deficiency chlorosis. N = marginal necrosis, leaves 3 and 4. IC = interveinal chlorosis, leaves 3 and 4. Severity of symptoms: See footnote Table 1.

(d) Tomato

Manganese toxicity in tomato is characterized by the development of iron deficiency chlorosis of the youngest leaves, and interveinal chlorosis of older leaves. Later, necrosis of the latter leaves occurs. This is usually first prominent in the distal leaflets in the form of discrete spots which may be either marginal or distributed over the lamina (Plate 6, Figs. 1, 2, and 3).

(i) *Radio-autographs*.—Individual leaves only were radio-autographed, the results being presented in Plate 6. These show that the greatest manganese concentration occurred in the terminal leaflet and the least in the basal leaflets. Individual leaflets were also higher in manganese at the tip than the base. Be-

fore the onset of any necrotic symptoms, the manganese was shown to accumulate in the interveinal tissues of the leaf (Plate 6, Fig. 4).

Later, marked localized accumulations of manganese, which corresponded with the development of necrotic spots, occurred (Plate 6, Fig. 1). Still later, on completion of the manganese-induced necrosis, this element actually migrates back into the veins and the necrotic spots themselves become very low in manganese (Plate 6, Figs. 2 and 3).

(ii) *Radio-assays*.—The results of radio-assays are presented in Table 5.

TABLE 5
TOMATO EXPERIMENT. RESULTS OF RADIO-ASSAYS FOR MANGANESE (P.P.M. DRY BASIS),
AND PLANT SYMPTOMS AFTER 14 DAYS IN CULTURE

Plant Part *	Mn 25 p.p.m.	Mn 25 p.p.m. Mo(NH ₄)† 20 p.p.m.	Mn 25 p.p.m. Mo (Na)† 20 p.p.m.
<i>Leaf 1</i>			
Leaflets	1960	1330	850
Petiole	3590	2790	1530
<i>Leaf 2</i>			
Terminal leaflet	2750	1340	1120
Centre leaflets	2330	1530	1230
Basal leaflets	1850	2770	1100
Petiole	1860	1320	570
<i>Leaf 3</i>			
Leaflets	2100	920	950
Petiole	1520	800	330
Symptoms‡	C++ N++	C+ N+	C++ N+

* The leaves are numbered from the base of the plant.

† $\text{Mo}(\text{NH}_4) = (\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$. $\text{Mo}(\text{Na}) = \text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$.

‡ C = iron deficiency chlorosis. N = necrosis (leaves 1 and 2).

These show that, in the absence of added molybdenum, the manganese distribution in the tomato leaf conforms in a general way with the results set out above with respect to the leaves of flax, peas, and cabbage. Thus, the highest manganese concentration occurred in the terminal leaflet, the middle leaflets were lower in manganese, and the basal leaflets contained the lowest concentration of this element. This also confirms the results of the radio-autographs (Plate 6, Fig. 1).

Where excess molybdenum (as ammonium molybdate) was added to the culture, the relative concentration of manganese in the leaflets of leaf 2 of the plants was reversed; i.e. the terminal leaflet had the lowest, and the basal leaflets the highest manganese concentrations. On the other hand, in the presence of excess sodium molybdate, manganese concentration was similar in each leaflet of the second leaf.

IV. DISCUSSION

The distribution of manganese within the plant has been shown to be similar for all the plant species studied. There was, in general, an accumulation of this element in the older rather than the younger tissues of the plant, and in the marginal or distal interveinal tissues of the leaves. Manganese was not accumulated by stem tissue to any great degree. With peas and flax, this characteristic pattern was the same for both normal and excess levels of manganese. However, with flax an important difference was noted, in that numerous minute "islands" of manganese occurred in the lower portions of the older leaves and stems at the normal level, whereas they were much less prevalent at the excess level. Bertrand and Rosenblatt (1922); Lyon, Beeson, and Ellis (1943); and Goodall (1943, 1949) have reported results similar to the above with respect to the existence of a gradient in manganese concentration between the older and younger leaves of plants. Jones and Bullis (1921) and Goodall (1945) have also shown that stems or petioles are much lower in manganese than the leaf lamina.

Manganese distribution in the plant, as determined in the present experiments, appears to be similar to that of molybdenum, which has been investigated by Lyon, Beeson, and Ellis (1943) and Stout and Meagher (1948). The latter, using radio-active molybdenum, showed that this element was not rapidly accumulated by actively metabolizing plant cells adjacent to the vascular tissue in the upper part of the plant, or by stem tissue. However, it was accumulated in the interveinal areas of the older leaves. However, Stout and Meagher state, without giving details, that molybdenum distribution in the plant was different from that of manganese. In this regard, however, Millikan (1947) has drawn attention to the general similarity between molybdenum deficiency and manganese toxicity symptoms in plants. Both these disorders are characterized by symptoms of interveinal chlorosis and marginal necrosis of the older leaves. Mulder (1948) has also described the occurrence of necrotic spots in the upper part of the leaflets of molybdenum-deficient tomato plants.

The literature indicating the existence of an interaction between manganese and molybdenum in plant nutrition has been reviewed by Millikan (1950), who also demonstrated the existence of highly significant but opposite interactions between manganese or molybdenum on the one hand, and nitrogen source on the other. It was further shown that the presence of ammonium ions in the solution significantly increased the toxicity to flax of a given excess of molybdenum. In the present experiments the addition of excess molybdenum in conjunction with excess manganese to a nitrate solution had important effects on both the concentration and distribution of manganese in the plant. In many instances a reduction in the manganese level in the tissue occurred. In some cases, the manganese distribution was quite abnormal in that the edge or terminal portion of the leaf contained a lower concentration than the remainder.

The movement of manganese after harvest, from the interveinal tissues into the veins, which has been demonstrated in the pea experiments above, indicates a possible limitation of the radio-autograph technique, in that a true pattern

of the distribution of the manganese in the living tissue may not be obtained. This migration of the manganese may have been encouraged by the fact that the plant was firmly pressed against the film between two sheets of glass while being radio-autographed. Thus evaporation of moisture through the leaf surface would be greatly reduced. It would appear evident that, to obtain radio-autographs giving the true location of manganese as it occurs in the living leaf, it is important to place the leaf in contact with the photographic emulsion as soon as possible after harvest. The radio-activity in the leaf should also be such as to reduce the time of exposure to a minimum. However, there is a possibility of radiation damage to the plants occurring if the radio-activity in the nutrient solution is increased. In this regard Russell (1949) found that even very low levels of radio-active phosphorus in water cultures may affect the rate of nutrient absorption and root growth.

An alternative to increasing the radio-activity in the plant to reduce the time of exposure for making radio-autographs would be to devise means of making radio-autographs of leaves without detaching them from the plant.

V. ACKNOWLEDGMENTS

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VI. REFERENCES

- BERTRAND, G., and ROSENBLATT, M. (1922).—Recherches sur les variations de la teneur en manganese des feuilles avec l'age. *Ann. Inst. Pasteur* **36**: 494-501.
- GOODALL, D. W. (1943).—Studies in the diagnosis of mineral deficiency. I. The distribution of certain cations in apple foliage in early autumn. *J. Pomol.* **20**: 136-43.
- GOODALL, D. W. (1945).—Studies in the diagnosis of mineral deficiency. II. A comparison of the mineral content of scorched and healthy leaves from the same apple tree. *J. Pomol.* **21**: 90-102.
- GOODALL, D. W. (1949).—Studies in the diagnosis of mineral deficiency. V. Manganese deficiency in wheat. *Ann. Appl. Biol.* **36**: 26-39.
- JONES, J. S., and BULLIS, D. E. (1921).—Manganese in commonly grown legumes. *J. Industr. Engng. Chem.* **13**: 524-5.
- LYON, C. B., BESSON, K. C., and ELLIS, G. H. (1943).—Effects of micro-nutrient deficiencies on growth and vitamin content of the tomato. *Bot. Gaz.* **104**: 495-514.
- MILLIKAN, C. R. (1947).—Effect of molybdenum on the severity of toxicity symptoms in flax induced by an excess of either manganese, zinc, copper, nickel or cobalt in the nutrient solution. *J. Aust. Inst. Agric. Sci.* **13**: 180-6.
- MILLIKAN, C. R. (1949).—Effects on flax of a toxic concentration of boron, iron, molybdenum, aluminium, copper, zinc, manganese, cobalt or nickel in the nutrient solution. *Proc. Roy. Soc. Vict.* **61**: 25-42.

- MILLIKAN, C. R. (1950).—Relation between nitrogen source and the effects on flax of an excess of manganese or molybdenum in the nutrient solution. *Aust. J. Sci. Res. B.* 3: 450.
- MULDER, E. G. (1948).—Importance of molybdenum in the nitrogen metabolism of micro-organisms and higher plants. *Plant and Soil* 1: 94-119.
- ODDIE, T. H., and MIBUS, S. A. (1949).—Data for assay of radio-active materials with geiger counter equipment. Commonwealth X-Ray and Radium Laboratory. Tech. Comm. No. 33.
- RUSSELL, R. S. (1949).—The problem of radiation damage when radio-active phosphorus is used in fertilizer experiments. *J. Chem. Soc. Supplementary Issue.* No. 2: S416.
- STOUT, P. R., and MEACHER, W. R. (1948).—Studies of the molybdenum nutrition of plants with radio-active molybdenum. *Science* 108: 471-3.

EXPLANATION OF PLATES 1-6

PLATE 1

- Fig. 1.—Radio-autographs of flax leaves, showing first symptoms of manganese-induced lower leaf necrosis, from plants grown in a nutrient solution containing 50 p.p.m. of manganese. Marked localized accumulations of manganese occur in the necrotic areas at the distal ends of the leaves.
- Figs. 2, 3, and 4.—Radio-autographs of flax plants grown in nutrient solutions for 8, 14, and 23 days respectively, the total manganese concentrations in the solutions in each case being: left—0.5 p.p.m., right—50 p.p.m. The accumulation of manganese in the distal halves of the leaves of the plant grown at both manganese levels is demonstrated. Small "islands" of manganese occur freely in the cotyledons and older leaves of the plants at the normal (0.5 p.p.m.) level at each time of sampling, but are only apparent to a very limited extent in the plants grown at the excess (50 p.p.m.) level.

PLATE 2

- Figs. 1, 2, and 3.—Radio-autographs of whole pea plants (one stipule from the base of each leaf removed) after 14 days in culture. Treatments: Figure 1. Mn 0.5 p.p.m.; Figure 2. Mn 25 p.p.m.; Figure 3. Mn 25 p.p.m. + Mo (as $(\text{NH}_4)_6 \text{Mo}_7 \text{O}_{24} \cdot 4 \text{H}_2\text{O}$) 20 p.p.m. Note the similarity of manganese distribution in the plant at normal (Fig. 1) and excess (Fig. 2) levels. The manganese concentration is much less in the youngest leaves and growing point than in the remaining leaves of the plant. Manganese accumulates in the marginal interveinal tissue of leaves and stipules, and in the tips of the tendrils. The stems and petioles are relatively low in manganese. With combined excesses of manganese and molybdenum (Fig. 3) the distribution of manganese throughout the entire plant is much more uniform than where excess manganese alone is applied (Fig. 2).

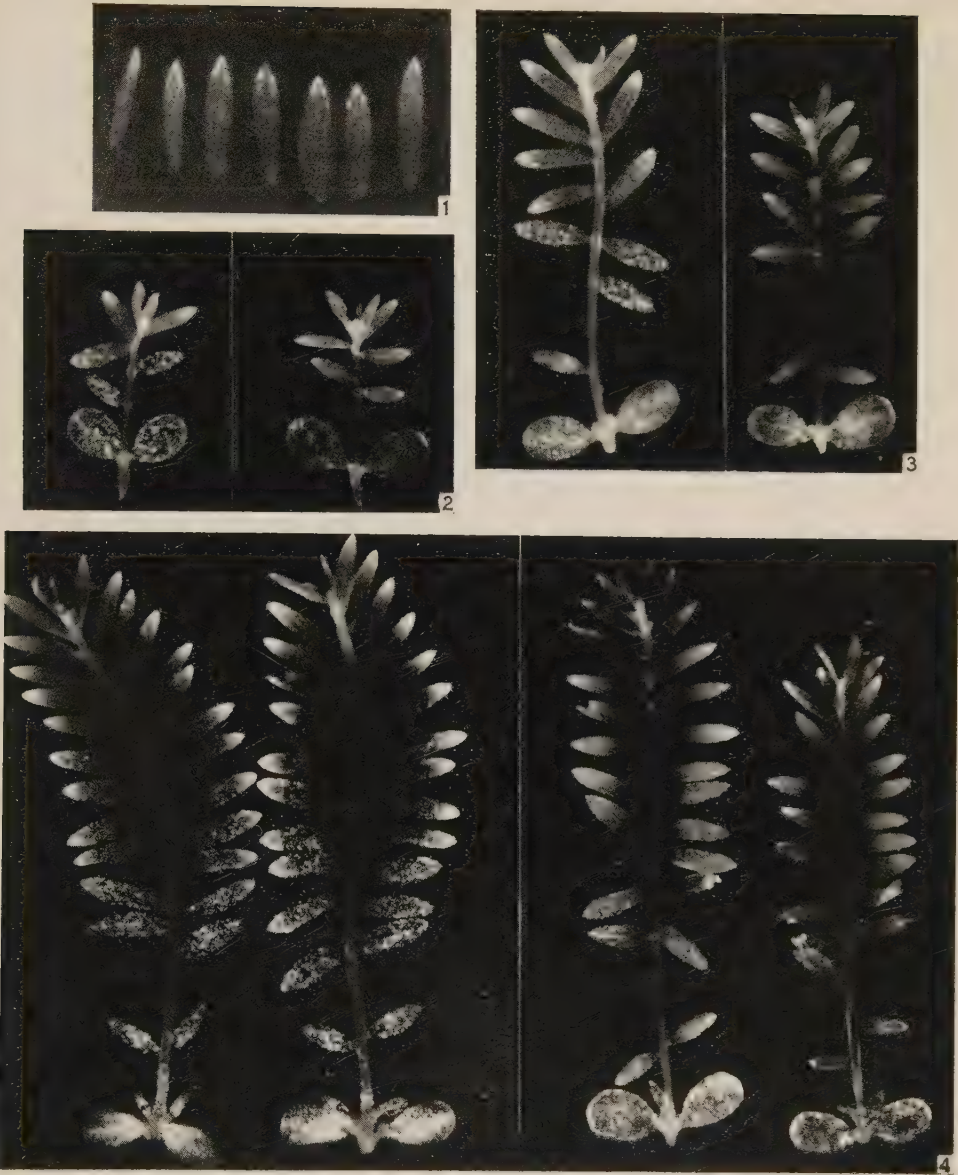
PLATE 3

- Figs. 1 and 2.—Radio-autographs of leaves from pea plants grown in nutrient solutions containing 25 p.p.m. of manganese, and sampled before the appearance of any necrosis. The manganese has accumulated in the marginal and interveinal (Fig. 1) tissues of the leaflets and in the tips of the tendrils.
- Figs. 3 and 4.—Left, photograph. Right, radio-autographs of leaves from pea plants grown in solutions containing 25 p.p.m. of manganese. The photographs show two stages in the development of manganese-induced necrosis of the marginal and interveinal leaf tissues. The radio-autographs of the same tissues reveal that there is actually a withdrawal of the manganese from the tissues as the necrosis becomes complete.

PLATE 4

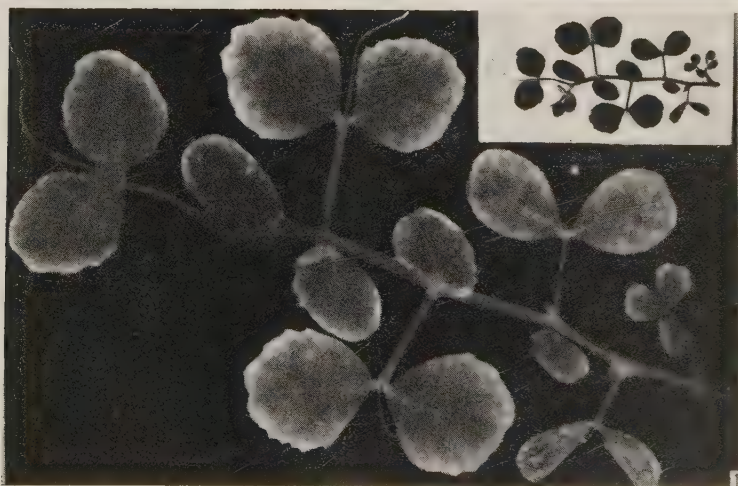
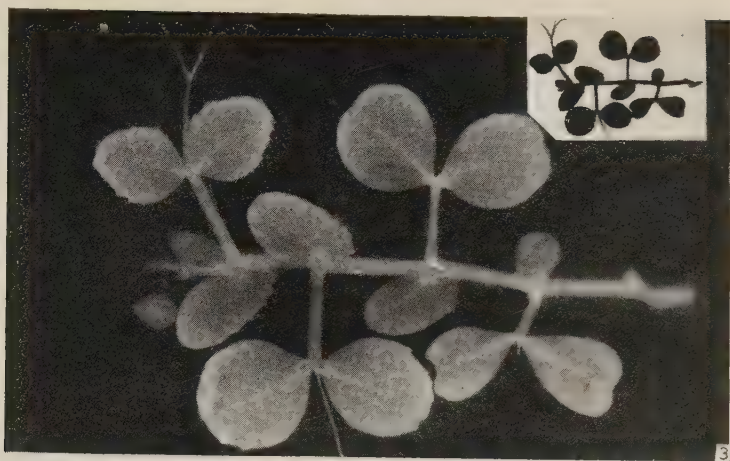
- Figs. 1 and 2.—Enlargements of successive radio-autographs of the same portions of pea plants receiving 25 p.p.m. of manganese. Left, first radio-autograph. Right, second radio-autograph. Time of exposure in each case twelve days. In the first radio-auto-

RADIO-AUTOGRAPHS OF MANGANESE





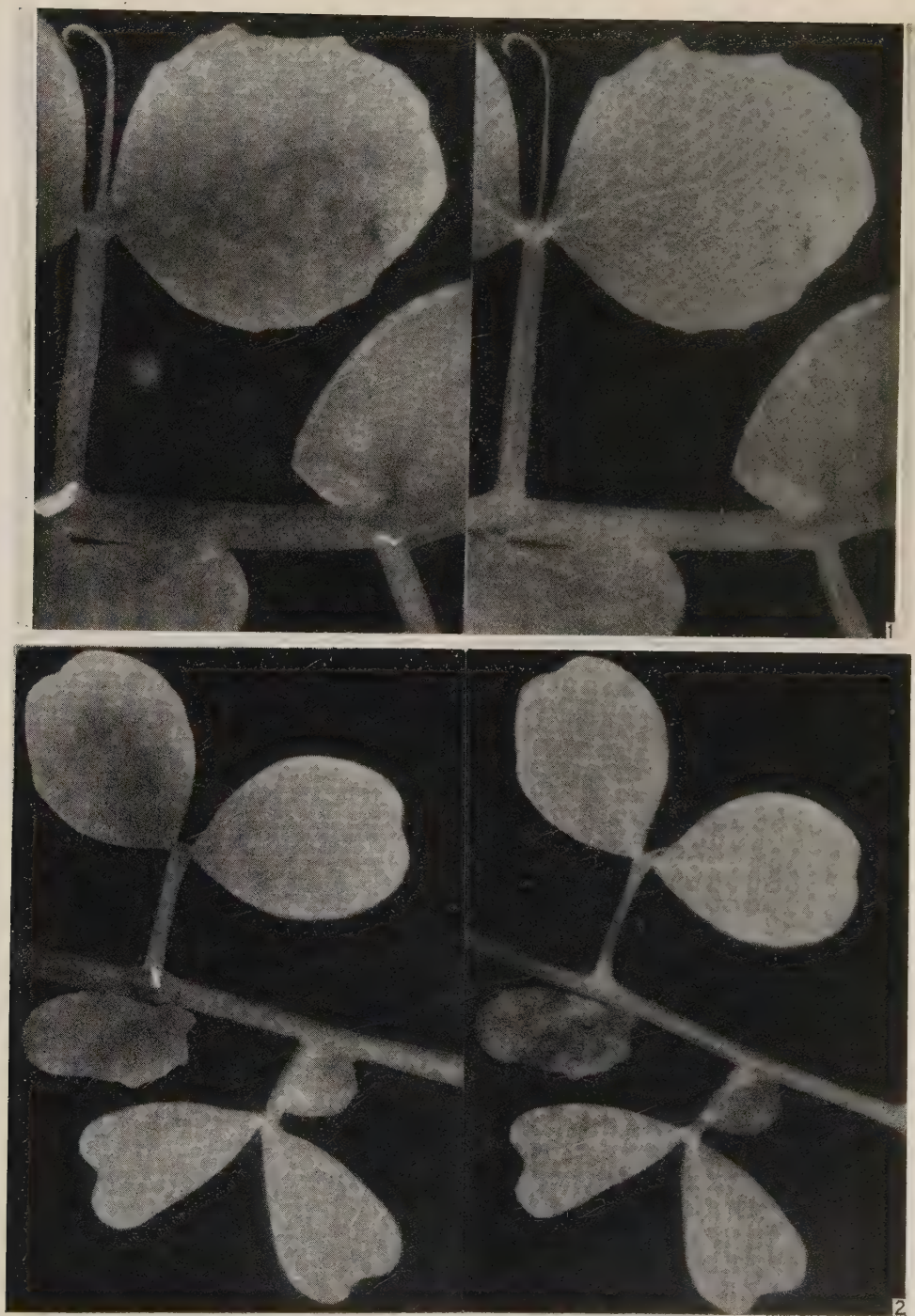
RADIO-AUTOGRAPHS OF MANGANESE



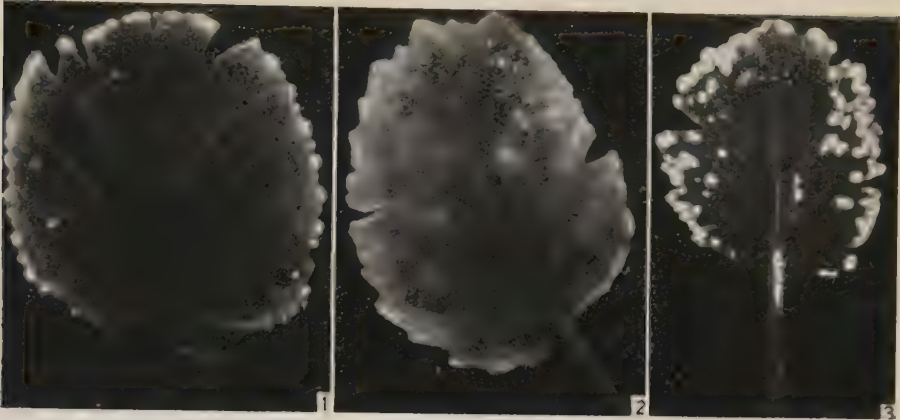
RADIO-AUTOGRAPHS OF MANGANESE



RADIO-AUTOGRAPHS OF MANGANESE



RADIO-AUTOGRAPHS OF MANGANESE



RADIO-AUTOGRAPHS OF MANGANESE



graphs the manganese is accumulated to a marked degree in the interveinal tissues of leaves and stipules, whereas in the second radio-autographs a pronounced redistribution of the manganese into the vascular tissues of leaflet, stipule, and petiole is indicated.

PLATE 5

- Fig. 1.—Radio-autograph of cabbage leaf showing manganese accumulation at the vein endings on the extreme margin of the leaf, indicating the probable excretion of the manganese through hydathodes.
- Fig. 2.—Radio-autograph showing the diffuse distribution of manganese in the interveinal tissues. When sampled the leaf showed symptoms of manganese-induced interveinal chlorosis in the same tissues.
- Fig. 3.—Radio-autograph of cabbage leaf showing the marked localization of manganese in areas corresponding to the initiation of necrotic spots.
- Fig. 4.—Enlargements of photograph (left) and radio-autograph (right) of half of cabbage leaf. The manganese accumulates in sharply defined spots which correspond with developing necrotic spots. The two necrotic spots indicated by arrows were the only ones that were fully developed (i.e. they were apparent on both leaf surfaces). Note that the manganese has moved out of the dead tissue of these two spots.

PLATE 6

- Figs. 1, 2, and 3.—Left, photographs. Right, radio-autographs of tomato leaves, showing symptoms of manganese-induced necrotic spotting, from plants grown in a nutrient solution containing 25 p.p.m. of manganese. In Figure 1 the upper half of the terminal leaflet was necrotic and manganese was becoming re-concentrated in the veins (compare Plate 6, Fig. 4). Necrotic spotting was just appearing in the lower leaflets and was preceded by or associated with marked localized accumulations of manganese.

The leaf in Figure 2 showed more advanced symptoms of manganese-induced necrotic spotting, the necrosis being apparent on both leaf surfaces. By contrast with Figure 1, no marked localization of manganese in association with the dead areas occurred, the element being concentrated instead in the veins. The enlargements of photograph and radio-autograph in Figure 3 of a terminal leaflet showing well-developed manganese-induced necrotic spotting, show that the actual necrotic areas ultimately become very low in manganese.

- Fig. 4.—Radio-autograph of terminal and adjacent leaflets of leaf from a tomato plant grown in a solution containing 25 p.p.m. of manganese. The leaf was sampled before the occurrence of any necrotic symptoms. The apical, marginal, and interveinal concentration of manganese in the leaflets is demonstrated. The base of the lower leaflet was inadvertently folded.

STUDIES ON THE DIGESTION OF WOOL BY INSECTS

I. MICROSCOPY OF DIGESTION OF WOOL BY CLOTHES MOTH LARVAE (*TINEOLA BISSELLIELLA* HUMM.)

By M. F. DAY

[Manuscript received August 28, 1950]

Summary

The larvae of *Tineola bisselliella* digest wool fibres. The scales, resistant to most enzymes, are, except for the epicuticle, as readily digested as are the cortical cells.

Partly hydrolyzed wool, as it occurs in fabrics or after weathering of raw wool, is digested. Raw wool, in which no sulphhydryl groups are present, is digested just as readily.

Visible evidence of digestion of keratin fibres may first be seen in a restricted section of the midgut and appears with dramatic suddenness when the passage of food is followed down the gut.

The epithelium of the region in which digestion is first visible differs histologically from the preceding region, and an unusually high reducing potential is maintained in the lumen in the same region.

I. INTRODUCTION

Significant work has been done on the physiology of digestion of wool by the larvae of the clothes moth (Linderstrom-Lang and Duspiva 1936), but the actual course of digestion is still under discussion, and it has never been determined whether the larvae are capable of digesting native wool with all its labile sulphur in the form of disulphide linkages. The changes in wool fibres during digestion have been described by Reumuth (1946) who has shown that the first sign of proteolysis of the wool is seen as longitudinal striations in the cortex of the fibres and that wool scales are as readily digested as are cortical cells. But Mandels, Stahl, and Levinson (1948), who studied the effects of certain bacteria and fungi on wool fibres, found that the microorganisms were unable to dissolve the scale cells. They concluded that "the evidence which indicates that solution does occur (as a result of digestion by any organism) is inadequate and needs confirmation."

The digestion of fibres by larvae of *Tineola* has been examined and Reumuth's observations have been extended in several respects. It is the purpose of this paper to provide the confirmation of his results required by Mandels, Stahl, and Levinson, and to complement these observations with new information on the physiology of the *Tineola* alimentary canal.

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II. MICROSCOPICAL OBSERVATIONS ON DIGESTION OF FIBRES IN THE TINEOLA GUT

The fibres are bitten into pieces of roughly equal length, averaging 70 to 150 μ , depending on the larval instar. When wool fragments are found in the foregut no signs of digestion can be seen in them. These fragments are "packeted," are passed through the unusually long oesophageal invagination, and thence to the midgut. Plate 1, Figures 1 and 2, shows photomicrographs with polarized light of wool fragments contained in the peritrophic membrane from the fore and hind parts respectively of the midgut of a *Tineola* larva. The fragments in the region just caudad to the oesophageal diverticulum are strongly birefringent (Plate 1, Fig. 1) and the scales are clearly visible (although only slightly so in the photograph). In the posterior part the fibres have lost much of their birefringence but are not swollen. Scales are not visible in the freshly dissected condition, after methylene blue staining, or after treatment with chlorine water (Allwörden reaction). Moreover, striations between the cortical cells are conspicuous, presumably due to the removal of some of the intercellular cementing substance (Plate 1, Figs. 2 and 3). Some isolated cortical cells may be observed in the lumen (Plate 2, Fig. 6). These changes in the ingested fragments are also made clear by appropriate staining. Methylene blue does not stain the fragments in the anterior part of the midgut. The further digestion has proceeded, the greater the distance the stain penetrates the cut ends of the fibres. Finally, the cortical cells all stain evenly. Results with Machida's stain (Glasgow 1934) are even more striking; the undigested fibres are yellow, whereas the separated cortical cells are red. Further details can be observed with the phase contrast microscope (Plate 2, Fig. 9), but clearer results are obtained with the peritrophic membrane and its contents than with the entire alimentary canal. In the hindgut, discrete fragments of wool fibres are usually not found, digestion having been complete. However, in larvae feeding rapidly, some fragments are not completely digested and may readily be seen in the centre of faecal pellets squashed in liquid under a coverglass (Plate 1, Fig. 4). This has been studied more fully by Mercer using electron microscopy (personal communication). Reumuth (1946) reported "droplike aggregations of digestive fluids" around the fibres in the gut. Although droplets may sometimes be seen in the gut lumen, no information has been obtained to confirm Reumuth's contention that they are concerned in digestive processes.

The fate of the scale cells is of particular importance. They are more resistant than cortical cells to *in vitro* enzymolysis (Hock, Ramsay, and Harris 1941). Chemically they probably contain a higher proportion of proline than does the remainder of the fibre (Lindley 1947), and this amino acid is thought to confer increased resistance to enzyme attack because of the folding it produces in the polypeptide chain. And indeed, scale cells are more resistant than the remainder of the fibre to attack by microorganisms (Mandels, Stahl, and Levinson 1948). In *Tineola* larvae, however, scales may be seen separating from the fibres about the middle of the midgut (Plate 2, Fig. 7), and in more

posterior regions no residue of scales can be discerned by light microscopy. Reumuth's contention that they are digested is thus unquestionably confirmed. Whether the epicuticle (described by Lindberg, Philip, and Graten 1948) is resistant has yet to be determined.

The digestion of wool fibres treated in various ways was examined. Thus, fibres in which 10 per cent. polymethacrylic acid had been deposited as an internal polymer, or 5 per cent. of the same reagent as an external deposit, or which had been treated with a melamine-formaldehyde resin, were digested just as the fibres from untreated fabric. Fabric treated by the method of Geiger, Kobayashi, and Harris (1942), to produce bithioether linkages in place of some of the disulphide groups, is rendered partially resistant to digestion by *Tineola* larvae. The gut of a larva fed such a fabric is full of wool fragments, and the only visible signs of digestion are in the striations in the cortex. The scales are intact even at the posterior end of the midgut. Wool treated by this method does not give the Allwörden reaction, and the scales of the wool fragments in the gut likewise fail to show changes microscopically in chlorine water.

The most striking feature of the change observed in the fibres in the *Tineola* larval midgut is its abruptness. This can be readily observed in the isolated midgut from any replete larva. It is illustrated in Plate 1, Figure 5, which shows marked changes in the properties of the fibres when viewed between crossed polars. Since the treatment necessary to produce, *in vitro*, fibres with the appearance of those shown below the arrow in Plate 1, Figure 5, consists of treatment with pepsin for some days (Hock, Ramsay, and Harris 1941), the effectiveness of the system *in vivo* is readily appreciated, especially when it is recognized that material passes completely through the alimentary tract within eight hours at 27°C. The structure and physiology of this region of the gut is, therefore, of considerable interest, and will be considered below (Sections IV and V).

III. THE ABILITY OF *TINEOLA* LARVAE TO DIGEST WOOL WITH ALL DISULPHIDE LINKAGES INTACT

Previous authors who have studied the digestion of wool have generally used fabric or weathered wool. In both of these, a proportion of the disulphide linkages, which are the basis of the resistance of keratin (Geiger, Kobayashi, and Harris 1942), are converted to sulphydryl groups.

In order to determine the effects of feeding wool keratin in which all disulphide linkages are intact, freshly shorn wool from a merino carrying a 3-in. fleece was thoroughly extracted in ether and in water. The absence of sulphydryl groups was determined in the half-inch nearest the skin by (1) the nitroprusside reaction, (2) absence of colour with triphenyltetrazolium chloride, (3) the phosphotungstic acid test in the hydrolysate produced by HCl in sealed tubes. This wool was resistant to digestion by trypsin or the proteases extracted from *Tineola* larvae. Microscopic examination of the contents of midguts of larvae of *Tineola* fed on this wool proved without question that they digested the fibres just as those fed on wool from fabrics (Plate 1, Figs. 1 and 2) in

which some of the disulphide linkages are reduced. Some of this sulphydryl-free wool was then partially reduced by calcium thioglycollate until it gave positive reactions with each of the above three tests; it was then found to be digested slowly *in vitro* by trypsin and by protease extracted from *Tineola* larvae.

IV. THE LOCALIZATION OF THE REDUCING REGION IN THE *TINEOLA* MIDGUT

Because of the extraordinary sharpness of the line in the midgut between wool showing no effects of digestion and the striated appearance described above, an attempt was made to find any characteristics of the gut that could be correlated with the ability to cause proteolysis in this region.

A useful indicator of reducing power is known in triphenyltetrazolium chloride (TTC) (Mattson, Jensen, and Dutcher 1947). When a midgut of a feeding larva is dropped into a 1 per cent. aqueous solution of TTC, a pink colour develops in the tissue within three minutes and is first usually apparent just at the zone where visible digestion of wool is seen. Later the colour spreads over the entire midgut and is found in some other tissues. For example, the body wall and the muscles give a positive reaction but the nerve cord, fat body, and the silk glands never do so. The midgut of larvae that have just emerged from the egg and have not yet fed gives no colour with TTC, nor does the midgut from a recently moulted larva. However, mature larvae continue to give an intense colour even after starvation for two days when no wool fragments remain in the gut, or after feeding from eclosion on an artificial diet containing no wool. The wool itself is negative but a faint colour can sometimes be seen within the peritrophic membrane. The main reaction is in the cytoplasm of the epithelial cells. It does not appear to be given by the contents of the goblet cells, but is given by their cytoplasm as well as by the cytoplasm of the columnar cells.

The nature of the reducing property of the larval midgut was studied by making up the TTC in 0.001M inhibitors and activators of enzyme systems, dropping the gut into the solution and determining the time taken for the appearance of the colour. In view of the similarity between a series of midguts of insects of the same age, this technique approximates the tissue slice method. The results are clear. Urea and cyanide are without effect, malonate slows the development of the colour, azide strongly inhibits it, whereas pyrophosphate strongly activates the reaction. These results suggest that a dehydrogenase is concerned in the maintenance of the reducing condition in the gut, and that it is most concentrated in the region of the gut most active in the digestion of wool fibres.

The localization of the reducing region in the midgut was confirmed in another way. Woollen fabric was reduced and cross-linked with mercuric cyanide by the method of Farnworth, Neish, and Speakman (1949) who believe that this treatment results in the incorporation of mercury between the sulphur atoms of the disulphide linkages. When such fabric is fed to *Tineola* larvae the wool is digested. The fabric is white in colour but the faecal pellets are black, probably due to the liberation of mercury following the rupture of the

-S-Hg-S- linkages. Further, the wool fibres in the gut are white in the anterior part of the midgut, but, at a point corresponding to the position where the TTC reaction is first observed, the fibres in the gut are blackened also, indicating precisely where reduction is taking place.

V. THE HISTOLOGY OF THE GUT IN RELATION TO DIGESTION

In view of the localization of strongly reducing conditions in the *Tineola* larval gut, it seemed desirable to determine whether these conditions were correlated with differences in the structure of the gut. Lotmar (1941) had already demonstrated that there are differences in histology in different parts of the midgut. However, it was not possible to correlate her observations with the TTC reaction until the two techniques were used on guts similar in size and history. It was found that good differentiation between the goblet cells and the columnar cells of the epithelium was obtained with Bodian's protargol technique following fixation in alcoholic Bouin's solution. It was immediately apparent that the reducing region of the midgut is poorly supplied with goblet cells whereas these are abundant anteriorly. The line between the two regions is very sharp (Plate 2, Fig. 10), and agrees exactly with the point where digestion of fibres becomes visible. Plate 2, Fig. 11, also shows the differences in form between the goblet cells of the two regions. Such differences have not been reported in the epithelium of other larval Lepidoptera. The posterior third of the midgut also contains abundant goblet cells and this region almost always gives a weak TTC reaction. It would thus appear that the columnar cells are responsible for the maintenance of the low potential.

Tineola larvae have been fed fabrics carrying a variety of dyestuffs. Certain dyes are released when the fibre is digested and may be absorbed. One such mixture of dyes* then sometimes appeared in the midgut epithelium as conspicuous needle-shaped crystals, which were metallic blue by reflected light and bright golden between crossed polars. These crystals occurred only in the columnar cells, and the line separating the region in which the crystals occurred coincided exactly with the point where digestion of the fibres became visible (Plate 2, Fig. 8). Woke (1941) has suggested that the goblet cells are especially concerned with the production of digestive enzymes. Since the columnar cells are concerned with absorption and with the maintenance of the oxidation-reduction potential, our results are not incompatible with Woke's hypothesis. There are differences in the frequency of occurrence of goblet cells along the length of a single region of a midgut. These differences are not constant from larva to larva from a single culture and indicate a cycle of secretory change presumably associated with phases in the processes of digestion.

* Said to be a commercial mixture of Alezarine brilliant blue PFN, fast light yellow 5GL, and acetyl rose 2GL. The individual dyes, which were kindly supplied by Hardie Trading Ltd., when applied to fabric did not, however, produce detectable crystals in the midgut epithelium.

VI. ACKNOWLEDGMENTS

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VII. REFERENCES

- FARNWORTH, A. J., NEISH, W. J. P., and SPEAKMAN, J. B. (1949).—The reactivity of the sulphur linkage in animal fibres. VI. The cause of unshrinkability. *J. Soc. Dy. Col. Bradford* **65**: 447-53.
- GEIGER, W. B., KOBAYASHI, F. F., and HARRIS, M. (1942).—Chemically modified wools of enhanced stability. *Bur. Stand. J. Res., Wash.* **29**: 381-9.
- GLASGOW, J. P. (1936).—Internal anatomy of a caddis (*Hydropsyche colonica*). *Quart. J. Micr. Sci.* **79**: 151-79.
- HOCK, C. W., RAMSAY, R. C., and HARRIS, M. (1941).—Microscopic structure of the wool fibre. *Bur. Stand. J. Res., Wash.* **27**: 181-90.
- LINDBERG, J., PHILIP, B., and GRATEN, N. (1948).—Occurrence of thin membranes in the structure of wool. *Nature* **162**: 458.
- LINDERSTROM-LANG, K., and DUSPIVA, F. (1936).—The digestion of keratin by the larva of the clothes moth (*Tineola bisselliella* Humm.). *C.R. Lab. Carlsberg* **21**: 53-83.
- LINDLEY, H. (1947).—Chemical constitution of keratin. *Nature* **160**: 190-1.
- LOTMAR, R. (1941).—Das Mitteldarmepithel der Raupe von *Tineola bisselliella* (Kleidermotte) insbesondere sein Verhalten während der Hautungen. *Mitt. schweiz. ent. Ges.* **18**: 233-48.
- MANDELS, G. R., STAHL, W. H., and LEVINSON, H. S. (1948).—Structural changes in wool degraded by the ringworm fungus *Microsporium gypseum* and other micro-organisms. *Text. Res. J.* **18**: 224-31.
- MATTSON, A. M., JENSEN, C. O., and DUTCHER, R. A. (1947).—Triphenyltetrazolium chloride as a dye for vital tissues. *Science* **106**: 294-5.
- REUMUTH, H. (1946).—The major textile pest—the moth. Moth-proofing of woollen materials in Europe. Textile Research Institute, Inc., N.Y.
- WOKE, P. A. (1941).—Structure and development of the alimentary canal of the Southern Armyworm larva. U.S. Dep. Agric. Tech. Bull. No. 762: 1-29.

EXPLANATION OF PLATES 1 AND 2

PLATE 1

Photomicrographs of fragments of wool fibres from larvae of *Tineola* photographed between crossed polars.

- Fig. 1.—Wool contained in peritrophic membrane immediately caudad to oesophageal invagination. Note strong birefringence of fibres. This and the following figure are from an insect fed native wool.
- Fig. 2.—Fibre fragments from the same peritrophic membrane from the region about two-thirds of the length of the midgut from the oesophageal invagination. Note loss of birefringence and striated appearance of fibres.
- Fig. 3.—Striated appearance of digested fibres from insect fed a fabric containing wool and some indigestible fibres. One of these indigestible fragments is seen at A.
- Fig. 4.—Macerated faecal pellet showing birefringent crystals (much of it is uric acid), and some partly digested wool fragments.
- Fig. 5.—Contents of anterior third of peritrophic membrane of *Tineola* larva showing at arrow the extraordinarily sharp line of demarcation between the fragments showing no signs of digestion and those in which digestion is obvious.

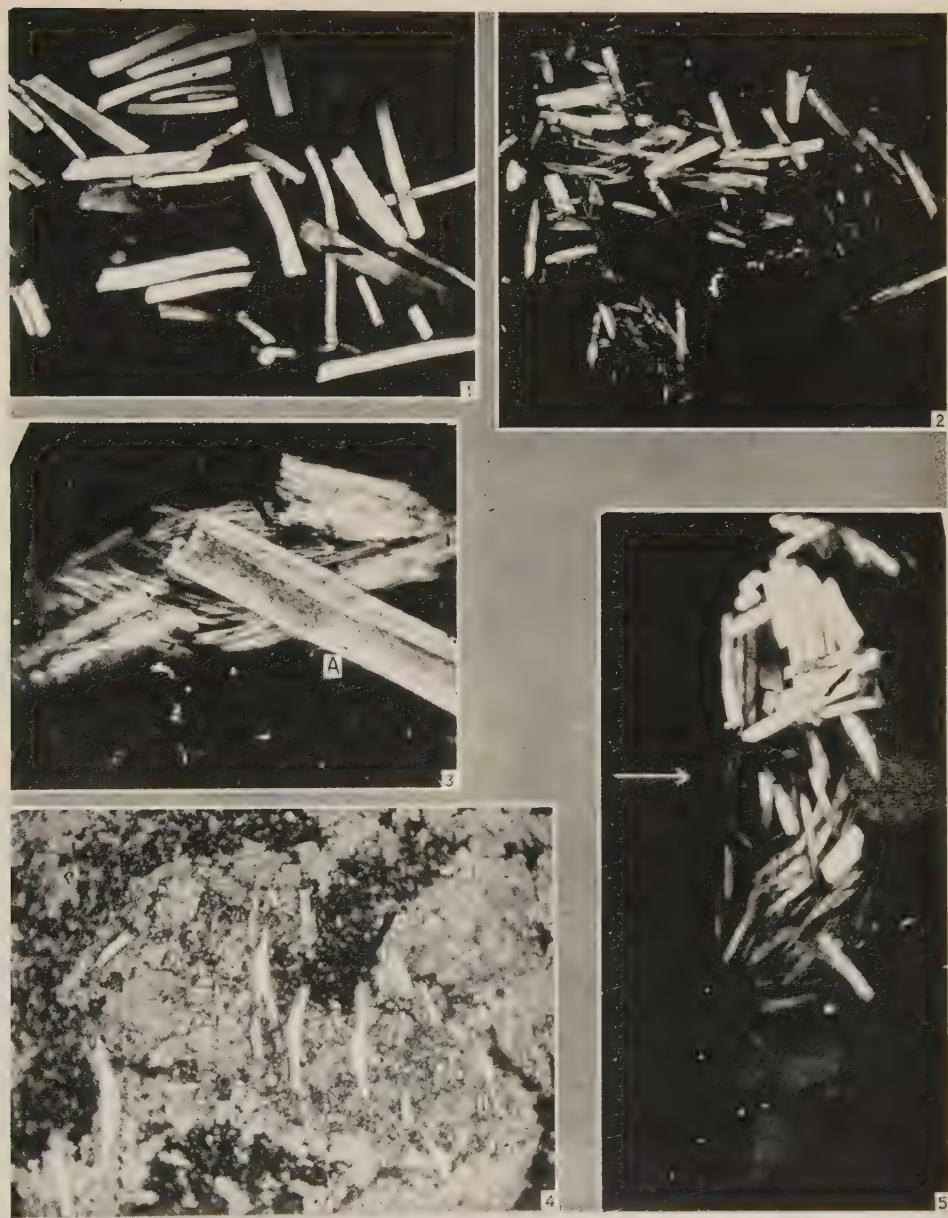
PLATE 2

- Fig. 6.—Partially digested fibre fragments from the midgut of a *Tineola* larva. Note the free cortical cells.
- Fig. 7.—The same as Figure 6. Note the scale cells (marked with arrow) separating from the fibres.
- Fig. 8.—Portion of midgut of *Tineola* larva fed a fabric containing certain dyestuffs. The dyes are released from the fabric on digestion, are absorbed by the midgut epithelium and produce crystals in the epithelial cells. Note the sharp line separating region where crystals are formed from the anterior portion of the midgut.
- Fig. 9.—Partially digested fibre fragments within *Tineola* peritrophic membrane under phase contrast.
- Fig. 10.—Longitudinal section of *Tineola* larval midgut showing sharp line between anterior portion in which goblet cells are abundant and region in which there are only occasional goblet cells among the columnar cells.
- Fig. 11.—Longitudinal section of *Tineola* larval midgut at later stage in digestion. Note wider striated border, the shape of the goblet cells, and the increase in numbers of regenerative cells at the base of the epithelium.

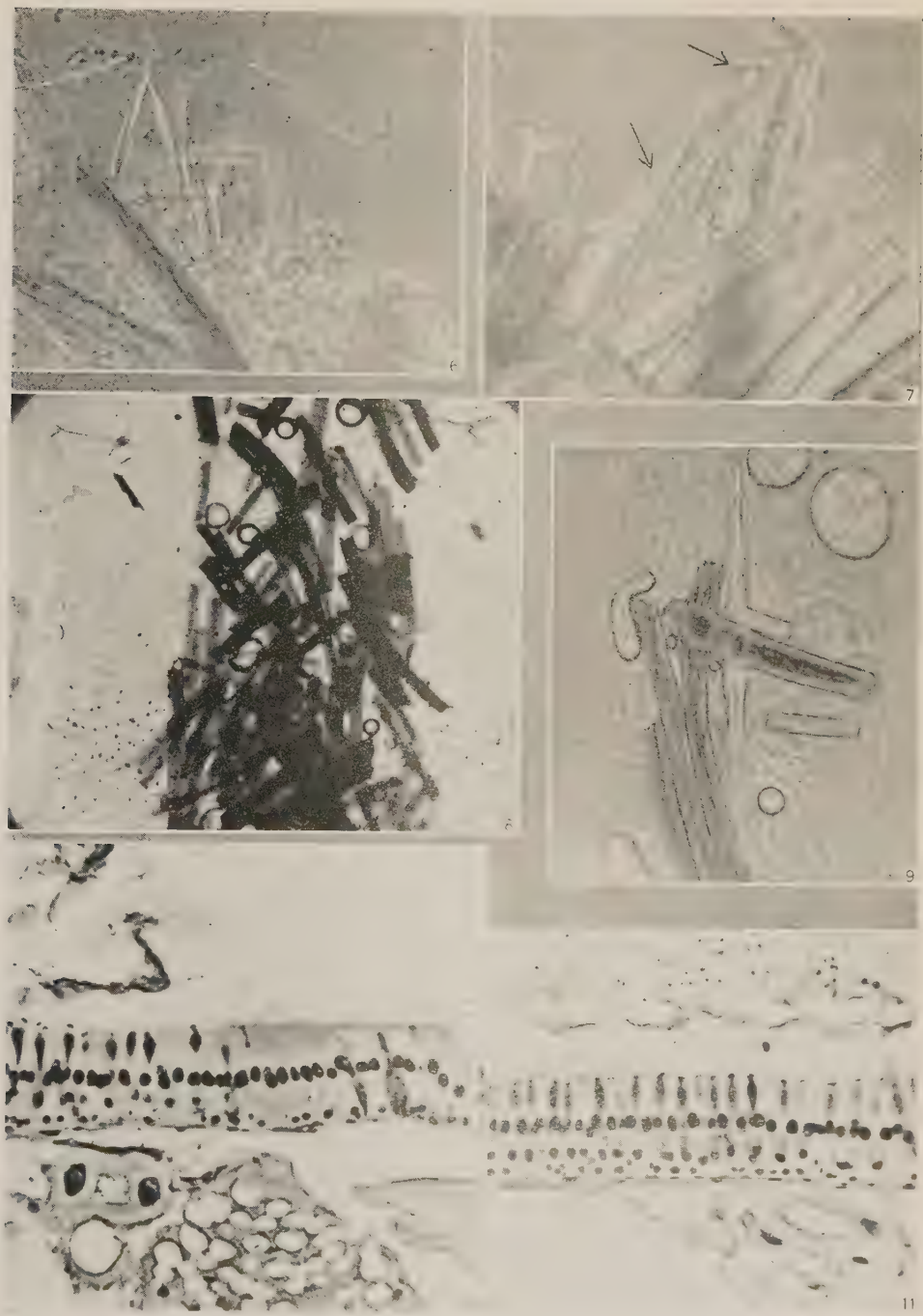
ADDENDUM

Experiments performed since the above was written have shown that the localization of the reducing conditions demonstrated by the use of an aqueous solution of triphenyltetrazolium chloride is not apparent if the dissection is performed beneath the fluid. Under these conditions, the most strongly reducing region is generally found to be the anterior part of the midgut. This observation suggests that the reducing conditions are rapidly destroyed upon exposure to the atmosphere. Although it is probable that the columnar cells are concerned with the maintenance of the reducing conditions, conclusions based on the localization of these conditions in the midgut must be revised. This will be discussed in greater detail in a subsequent paper in this series.

DIGESTION OF WOOL BY INSECTS. I



DIGESTION OF WOOL BY INSECTS. I



STUDIES ON THE DIGESTION OF WOOL BY INSECTS

II. THE PROPERTIES OF SOME INSECT PROTEINASES

By R. F. POWNING,* M. F. DAY,* and H. IRZYKIEWICZ*

[Manuscript received August 28, 1950]

Summary

The effects of a number of protease activators and inhibitors, namely cyanide, iodoacetate, thioglycollate, enterokinase, ovomucoid, *Ascaris* inhibitor, and soybean inhibitor, have been studied on crude proteinase preparations from the midguts of the following five species of insects: *Tineola bisselliella* larvae, *Tenebrio molitor* larvae, *Musca domestica* larvae, *Periplaneta americana*, and *Locusta migratoria*, and the results compared with their effects on trypsin and papain. Similarly, a comparison has been made of the pH optima, heat stability, and milk-clotting ability of these enzyme preparations and of the effects of oxidation-reduction potential on their hydrolytic ability. In general, the insect enzymes are similar, and resemble trypsin more closely than papain. Those differences that are found between enzymes from the sources mentioned indicate that the special ability of the larva of the clothes moth *Tineola* to digest keratin does not reside in its protein-digesting enzymes.

I. INTRODUCTION

It is generally considered that "the digestive enzymes of insects resemble those of other animals; they are activated and inhibited by the same reagents, they are similarly affected by changes in pH and so forth" (Wigglesworth 1939). However, Linderstrom-Lang and Duspiva (1936) showed that the proteinase of *Tineola* larvae differed from vertebrate pancreatic trypsin in that it was not inhibited by sulphhydryl-containing compounds, and they considered this to be a useful adaptation to the substrate because of the high concentration of cystine in the diet of clothes moths. Duspiva (1936) found that the proteinase of the larva of the wax moth *Galleria* reacted to thioglycollic acid in a manner intermediate between *Tineola* proteinase and trypsin. The question has remained unanswered whether these lepidopterous larvae differ from other insects in their comparative insensitivity to sulphhydryl compounds, or whether all insect proteinases differ from trypsin in this respect.

In order to answer this question, and to determine whether the digestive proteinase of *Tineola* differs from those of other insects in other features, which might account for its peculiar ability to digest keratin, the experiments reported below were undertaken. It was found that the differences between insect proteinases, although demonstrable, were not marked. However, a number of exceptions to Wigglesworth's generalization, quoted above, became apparent.

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II. METHODS

(a) *Proteinase Preparations*

Proteinase preparations were obtained from adults of *Periplaneta americana* (L.) and of *Locusta migratoria* (L.); larvae of *Tenebrio molitor* L., larvae of *Musca domestica* (L.), and larvae of *Tineola bisselliella* Humm. These were prepared for the experiments with activators and inhibitors by homogenizing the freshly dissected midguts and contents in 0.067M phosphate buffer at pH 8.0. Only caeca were used with *Periplaneta* and *Locusta*. These preparations were compared with trypsin (B.D.H.) and papain (commercial preparation). Details of a typical series of these preparations are given in Table 1.

TABLE 1
DETAILS OF ENZYME PREPARATIONS FOR INHIBITOR EXPERIMENTS

Source of Enzyme	Number of Insects/ml.	Proteinase Activity (optical density)	Protein N (mg./ml.)
<i>Periplaneta</i>	0.6	0.271	0.048
<i>Locusta</i>	0.024	0.264	0.089*
<i>Tenebrio</i>	4.25	0.238	0.848
<i>Musca</i>	7.5	0.263	0.248
<i>Tineola</i>	47	0.233	0.272
Trypsin	0.310†	0.270	0.033*
Papain	7.45†	0.248	0.252

* Total nitrogen.

† Mg./ml.

For the examination of the effect of heat on the enzymes, a phosphate-borate buffer at pH 6.0 was used for papain, a carbonate-borate buffer at pH 10.0 for *Tineola*, and phosphate-borate buffer at pH 8.0 for the other enzymes. For all other experiments, water extracts of acetone-dried material were used.

(b) *Activators and Inhibitors*

(i) *Proteinase Determinations.*—These were performed by the method of Charney and Tomarelli as described by Day and Powning (1949), except that half the quantity of double concentration of azocasein substrate was used to permit addition of reactants.

(ii) *Activators and Inhibitors of Proteases.*—There were prepared as follows:

(1) Sodium cyanide—0.5M solution adjusted to pH 8.0 with HCl.

(2) Thioglycollic acid—neutralized by slow addition of NaOH while cooling in an ice bath. The thioglycollic acid was added as a 0.45M solution, but, owing to the tendency to rapid oxidation, the concentration was reduced. Its effect on papain, however, indicated that it was an active preparation.

(3) Sodium iodoacetate—0.5M solution adjusted to pH 8.0 with NaOH.

(4) Ovomucoid—prepared according to the trichloroacetic acid-acetone precipitation method of Lineweaver and Murray (1947).

(5) Enterokinase—prepared by water extraction from recently acetone-ether-dried pig intestinal mucosa (Hawk, Oser, and Summerson 1947, p. 360).

(6) *Ascaris* inhibitor—prepared according to the shorter method of Collier (1941).

(7) Soybean inhibitor—prepared by extraction at pH 4.2 and purified with kaolin and acetone (Ham and Sandstedt 1944).

(c) *pH Optima*

An attempt was made to use the azocasein method for this work, but it was discarded in favour of the gelatin viscosity method of Lennox (1943). The enzyme concentrations were adjusted to give about 15-20 per cent. reduction in viscosity. Five ml. of 10 per cent. gelatin at the required pH was mixed with 1.8 ml. water in a viscometer and kept in a water bath at 35.5°C. Viscosity readings (outflow times) were taken, 0.2 ml. enzyme was added and after a digestion period of 15 minutes a further viscosity reading was taken. The amount of digestion at the various hydrogen ion concentrations was plotted as percentage reduction in viscosity. A correction was made on each figure for the dilution by the enzymes.

(d) *Milk Clotting*

Milk clotting was studied by the method of Balls and Hoover (1937). All enzymes were adjusted to the same concentration by estimation with the azocasein proteinase method at pH 8.0.

(e) *Heat Stability*

Proteinase determinations were carried out using the normal concentration azocasein substrate adjusted to the same pH as the enzyme solutions. Aliquots of 0.5 ml. of enzyme solution were heated in tubes in a water bath for various times at temperatures varying from 60°C. to boiling, then, on removal and cooling, 0.5 ml. of substrate was added and the tubes incubated at 37°C. for 1 hr. Curves were drawn relating residual activity to time and temperature of heating and the times for the destruction of one-half of the activity reported.

(f) *Oxidation-Reduction Potential*

(i) *Consideration of Methods*.—Considerable difficulty was experienced in developing a satisfactory method for the examination of the effects of oxidation-reduction potential on proteinase activity. The problem consisted, firstly, of establishing and measuring a range of stable oxidation-reduction potentials in the digestion mixture, and secondly, of estimating the amount of digestion. An attempt was made to use the electrolytic technique of Hanke and Katz (1943). The oxidation-reduction potential was maintained at various levels under nitrogen by direct current electrolysis using a platinum anode or cathode, depending upon whether a high or low potential was required. The method was found to be impracticable under our conditions owing to the very poor poisoning in the

solutions and the rapid changes in pH that occurred with electrolysis. A technique using partially reduced dyes as poisoning agents and a formol titration for digestion failed because of the difficulty of keeping the oxidation-reduction potential constant for periods of time longer than one hour.

The technique that yielded constant oxidation-reduction potentials and a minimum reaction time was one in which $\text{Na}_2\text{S}_2\text{O}_4$ was added to a gelatin substrate to adjust the oxidation-reduction potential to a value at which an appropriate oxidation-reduction indicator in the solution was partially reduced, and reduction in gelatin viscosity taken as measure of digestion.

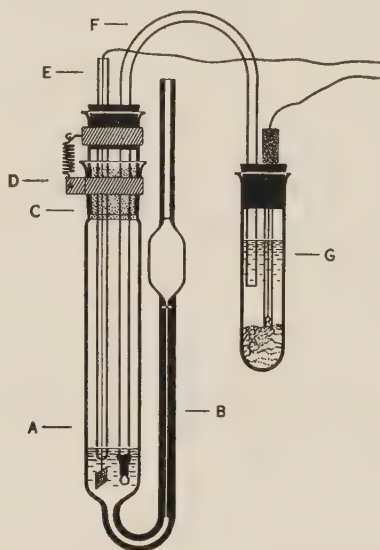


Fig. 1.—Diagram of reaction vessel for determining effect of oxidation-reduction potential on proteolysis. A, reaction vessel; B, viscometer; C, ground-glass joint; D, spring clip; E, platinum electrode; F, KCl bridge; G, calomel half cell. Mixing of the contents of vessel A was accomplished by bubbling air or nitrogen, depending on whether high or low oxidation-reduction potentials were required.

The reaction vessel (Fig. 1) was a Pyrex tube 20 cm. long and 2.5 cm. diameter. The bottom of the vessel was sealed to a viscometer side-arm of about 3 ml. capacity and having an outflow time of about 25-30 seconds for water at 35.5°C . The vessel was attached by a ground-glass joint to an adjustable stand, which could be lowered into an oil bath at 35.5°C . A platinum electrode and salt bridge from a calomel electrode were permanently mounted in the stand for oxidation-reduction potential measurements. The procedure was as follows: 5 ml. of diluted gelatin at pH 8.0 and 0.5 ml.

of dye solution were pipetted into the reaction vessel and warmed to 35.5°. Readings of outflow time were taken to check the constancy of the gelatin viscosity. Sufficient freshly prepared 0.1M $\text{Na}_2\text{S}_2\text{O}_4$ solution was added to convert half the dye to the reduced form, and the total volume of the mixture was made up to 5.8 ml. with water. Then 0.2 ml. of enzyme solution was added and the mixture was stirred with purified nitrogen.

Viscosity readings were taken after 5 and 15 minutes digestion. Proteinase activity was measured as percentage reduction of gelatin viscosity after 15 minutes. A correction was made to all results to account for the dilution effect of adding the $\text{Na}_2\text{S}_2\text{O}_4$ and enzyme by subtracting the reduction in viscosity resulting from the addition of an equivalent quantity of water. A control test with water only was carried out for each enzyme. The data are reported as differences between activity of the test mixtures and of controls containing no poisoning system.

The gelatin substrate was purified and prepared as a 12 per cent. stock solution according to Waksman and Davison (1926). This was kept in small quantities preserved with thymol in the refrigerator. When required, the stock solution was diluted to 3 per cent. with 0.067M phosphate buffer at pH 8.0 and kept overnight at 35.5°C. to stabilize the viscosity.

Oxidation-reduction potential readings were taken with a Cambridge potentiometer at the same time as the viscosity measurements. They were generally constant within ± 1 mv. throughout the digestion period; in a few tests, however, they varied up to ± 20 mv.

(ii) *Poising Systems Used.*—The following dyes were chosen:

Dyes	E_o (mv.)
Disodium 2,6-dibromobenzenoneindo-3-carboxyphenol	+ 250
1-Naphthol-2-sodium sulphonate-indophenol	+ 123
Methylene blue	+ 11
Potassium indigodisulphonate	— 120
Rosinduline	— 281
Benzyl viologen	— 359

(iii) *Adjustment of Oxidation-Reduction Potential of Gelatin with Other Reactants.*—Further experiments were carried out using other inorganic oxidizing and reducing agents to give a wide range of oxidation-reduction potentials. The reactants used were 0.1M $\text{Na}_2\text{S}_2\text{O}_4$, 0.1M $\text{K}_2\text{S}_2\text{O}_8$, 0.1M $\text{K}_3\text{Fe}(\text{CN})_6$. The technique was essentially the same as described for the dyes; 5 ml. of gelatin and 0.8 ml. of reactant were mixed, the initial pH and viscosity checked, and then 0.2 ml. of enzyme was added. Control tests were carried out on each enzyme by replacing the oxidizing or reducing agents by water. The enzyme activity was measured as percentage reduction in gelatin viscosity after a 15-minute digestion period.

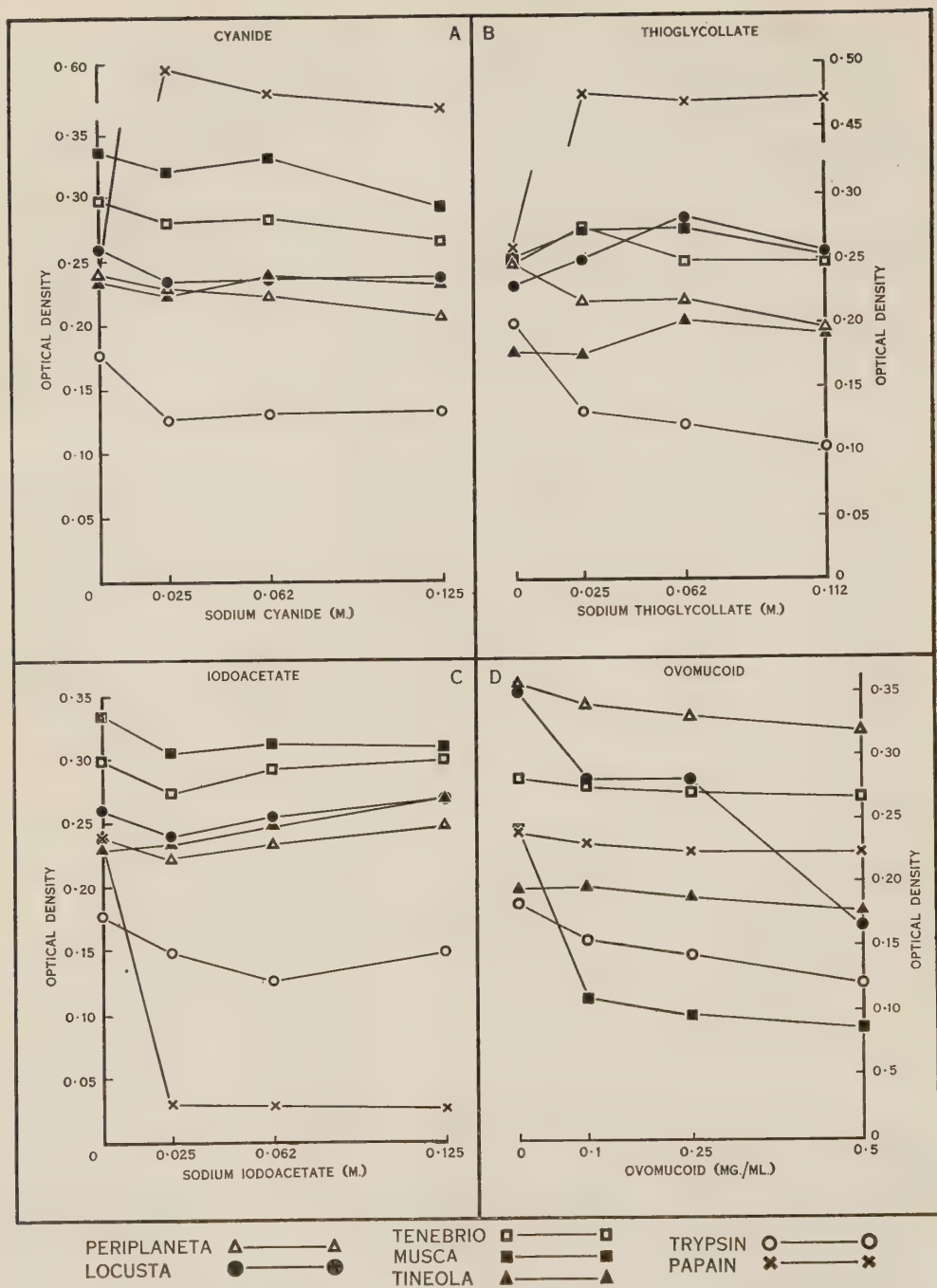


Fig. 2.—Effects of activators and inhibitors on proteinases.

III. OBSERVATIONS

(a) *Effects of Activators and Inhibitors on Proteinases*

The results are presented in Figures 2A-D and 3A and B. The concentrations used were those effective on trypsin and papain.

(i) *Cyanide* (Fig. 2A).—Papain was activated markedly, trypsin was slightly inhibited, but all the insect enzymes were unaffected.

(ii) *Thioglycollate* (Fig. 2B).—It is clear from the results that, at the concentrations employed, thioglycollate activated papain strongly, and inhibited trypsin. It had comparatively little effect on the insect enzymes.

(iii) *Iodoacetate* (Fig. 2C).—Iodoacetate strongly inhibited papain, but trypsin and the insect enzymes were not markedly affected.

(iv) *Ovomucoid* (Fig. 2D).—As was expected, trypsin was strongly inhibited. However, there are certain surprising differences in the reactions of the different insect enzymes. Those from *Locusta* and *Musca*, for example, were both inhibited, but those from *Tineola*, *Periplaneta*, *Tenebrio*, and papain were unaffected.

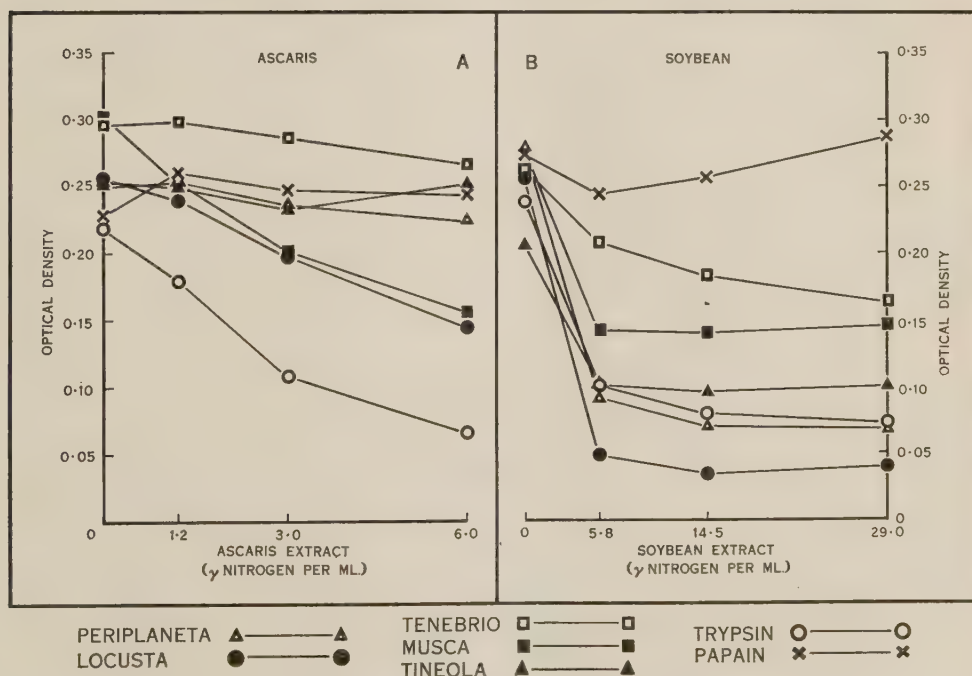


Fig. 3.—Effects of inhibitors on proteinases.

(v) *Enterokinase*.—The effect of enterokinase on trypsin was only slight, but this was due to the fact that the trypsin used was almost fully activated. However, a confirmatory test using the same enterokinase and trypsin prepared

from freshly acetone-ether-dried pancreas showed that the enterokinase was highly active; it caused an increase in activity of this trypsin from 0.099 to 0.550 units of optical density. Enterokinase had no effect on papain or on any of the insect enzymes tested.

(vi) *Ascaris Inhibitor* (Fig. 3A).—This inhibitor gave similar results to those of ovomucoid—trypsin, *Musca*, and *Locusta* were inhibited, while the other enzymes were not.

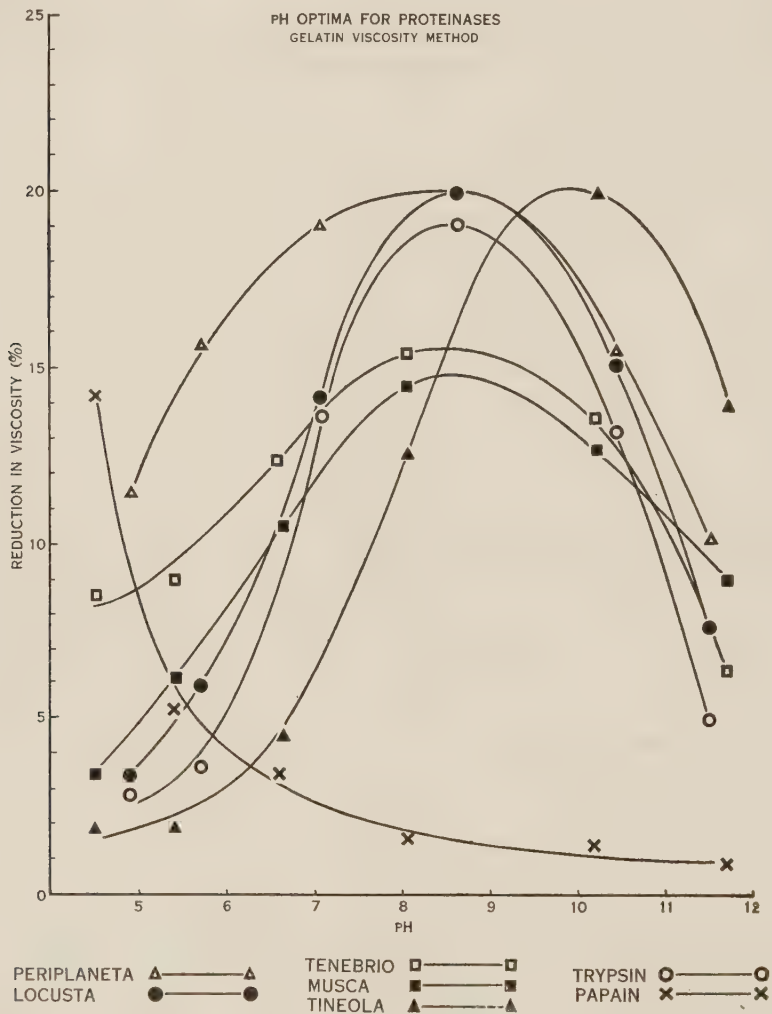


Fig. 4.—Effects of pH on proteinases.

(vii) *Soybean Inhibitor* (Fig. 3B).—Papain was unaffected by the soybean extract at the concentration used, but all the other enzymes were inhibited, to varying degrees. The enzyme from *Tenebrio* was inhibited least and that from *Locusta* to the greatest extent.

(b) Effect of pH on Proteinases

The curves in Figure 4 show that, with the gelatin method under the conditions used, trypsin had an optimum pH of about 8.5 and, as expected, papain had its greatest activity at a pH lower than 6. Measurements were not made below pH 4.5. Of the insect enzymes, that from *Tineola* is outstanding as its optimum is about pH 9.8-10, and its activity rapidly falls off below this. All the other insect proteinases have the same pH optimum as trypsin. However, the enzymes from *Periplaneta*, *Musca*, and *Tenebrio* have a wider range of activity than those from the other insects examined.

TABLE 2
MILK-CLOTTING ACTIVITY OF PROTEASES

Source of Enzyme	Action on Azocasein at pH 8.0 (optical density)	Action on Milk at pH 4.6 (mean clotting time, min. and sec.)
<i>Periplaneta</i>	0.25	5-44
<i>Locusta</i>	0.29	7-25
<i>Tenebrio</i>	0.26	4-52
<i>Musca</i>	0.29	15-5
<i>Tineola</i>	0.29	103
Papain	0.27	6-42
Trypsin	0.29	52

(c) Milk-Clotting Activity of Proteases

When standardized with the azocasein method at pH 8.0 to the same proteinase activity, the ability of the various enzymes to clot milk at pH 4.6 differed (Table 2). The enzymes had the same order of activity, with the excep-

TABLE 3
TIME IN MINUTES FOR DESTRUCTION OF HALF ENZYME ACTIVITY AT VARYING TEMPERATURES

Source of Enzyme	pH of Extract	Temperature				
		98°C.	90°C.	80°C.	70°C.	60°C.
<i>Periplaneta</i>	8.0	<1	<1	<1	1½	23
<i>Locusta</i>	8.0	<1	<1	<1	<1	13
<i>Tenebrio</i>	8.0	<1	<1	<1	2	4
<i>Musca</i>	8.0	<1	<1	<1	<1	15
<i>Tineola</i>	10.0	<1	<1	<1	<1	1
Trypsin	8.0	<1	<1	<1	1½	3
Papain	6.0	<1	1¾	13	120	510

tion of the enzyme from *Tineola* and of trypsin, which clotted milk very slowly. This apparently anomalous behaviour of the *Tineola* proteases undoubtedly can be explained by the differences in pH optimum (cf. Fig. 4).

(d) Heat Stability of Proteinases

Although the enzyme solutions were crude extracts, and conclusions based on small differences were unwarranted, it is clear from Table 3 that papain is much more resistant to thermal inactivation than any of the other enzymes.

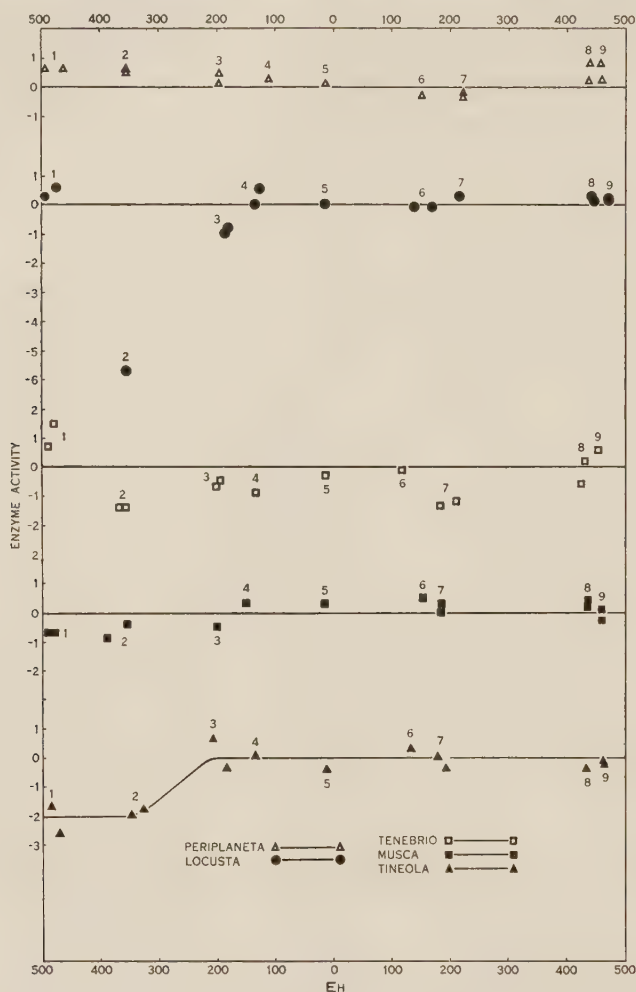


Fig. 5.—Effects of oxidation-reduction potential on proteinases. Numbers on the graphs refer to poisoning systems, which are as follows: 1, sodium hydrosulphite; 2, benzyl viologen; 3, rosinduline; 4, potassium indigodisulphonate; 5, methylene blue; 6, 1-naphthol-2-sodium sulphonate-indophenol; 7, disodium 2, 6-dibromobenzenoneindo-3-carboxyphenol. 8, potassium persulphate; 9, potassium ferricyanide.

Enzyme activity in the absence of a poisoning system is taken as zero. Differences between means are significant at the 1 per cent. level when they exceed 1.6 units, and at the 5 per cent. level when they exceed 1.2 units.

Tineola proteinase is similar to trypsin and to the other insect enzymes, except that from *Periplaneta*. This enzyme appears to be slightly more resistant to heat.

(e) Effects of Oxidation-Reduction Potential on Proteinases

The data from a large number of determinations of the effect of oxidation-reduction potential on proteolytic activity are summarized in Figures 5 and 6. The results are reported as enzyme activity in the treated samples relative to the activity of the water controls. It will be observed that the Eh varied from about - 460 to + 460 mv. Although the physiological range was covered, only relatively slight changes in activity of the insect proteinases and of trypsin were demonstrated by the methods employed.

The apparent effect of methylene blue on papain was found to be the result of poisoning. This was demonstrated by the effect of different concentrations at the same potential (Table 4).

TABLE 4

EFFECTS OF INCREASED CONCENTRATIONS OF METHYLENE BLUE ON PAPAIN ACTIVITY

Final Concentration of Methylene Blue (%)	Eh (mv.)	Activity (%)
0.0083	- 14	54.6
	- 10	49.4
0.00083	- 12	64.9
	- 4	64.5

Benzyl viologen probably poisoned *Locusta* proteinase, as indicated by the fact that $\text{Na}_2\text{S}_2\text{O}_4$, giving a lower potential, did not reduce the activity of this enzyme.

IV. DISCUSSION

In the work described above it would have been preferable to use purified enzyme preparations. This, however, was not practicable because of the large quantity of material required for purification. It was considered that sufficient indication of the enzyme properties could be obtained from crude extracts. The results with these extracts will be considered in relation to work on enzymes from other sources.

(a) Effects of Activators and Inhibitors

The failure of cyanide and thioglycollate to activate insect proteinases, and of iodoacetate to inhibit them, differentiates these enzymes sharply from many plant proteases. The absence of inhibition by cyanide and thioglycollate, and of activation by enterokinase, also differentiates them from trypsin of vertebrate origin. Schlottke (1937*b*) has claimed a small degree of activation by enterokinase of proteinase from *Carabus* and *Periplaneta*. On the other hand, the observation that trypsin is 25 times more sensitive to ovomucoid than any other proteinase studied by Fraenkel-Conrat, Bean, and Lineweaver (1949) suggests that those proteinases from insects that are similarly sensitive are closely related to trypsin in this respect.

Like the plant proteinases, those from insects have not been found capable of further activation and we cannot account for the discrepancy between our results and those reported for *Periplaneta* by Schlottke (1937*b*) with respect to enterokinase.

The inhibition of the proteinases from *Musca* and *Locusta* by ovomucoid and the *Ascaris* inhibitor suggests a similarity in mode of action of these two substances, and indicates that some differences exist between the enzymes from different insects.

Similarly, the inhibition of *Carabus* and *Tettigonia* proteinases by cyanide and cysteine (Schlottke 1937*a*) shows that the enzymes from these species differ from those of the species studied in the present paper. Additional differences between insect proteinases are suggested by the other factors studied.

(b) pH Optima

The pH optima of the insect proteinases examined vary somewhat from figures previously published (Table 5). All the values reported from the literature were determined by formaldehyde titration, except that for *Tineola* larvae, for which the Willstätter alcohol titration was used.

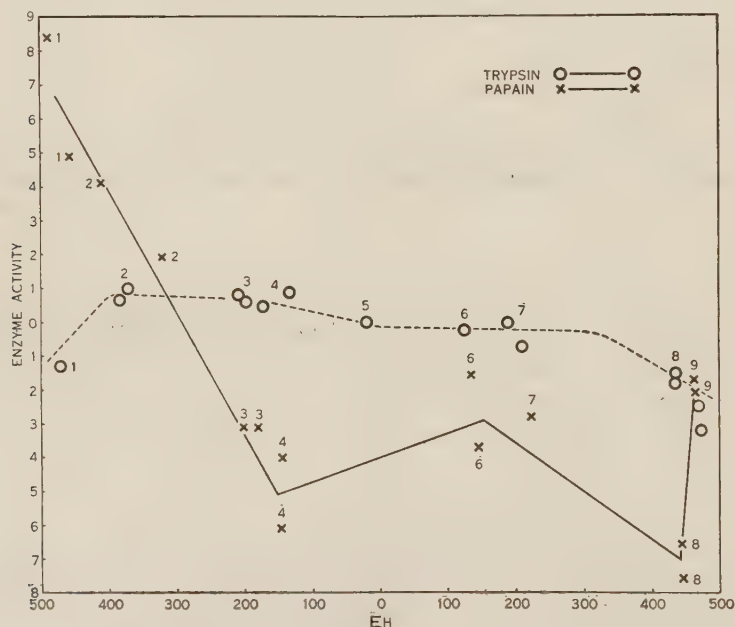


Fig. 6.—Effects of oxidation-reduction potential on proteinases. Details as in Figure 5.

These differences may all probably be accounted for by the differences in methods employed. However, under standardized conditions, the pH optima of the insect proteinases studied do demonstrate a difference between the enzyme from *Tineola* and other insects; this difference cannot be due to the impurity

of the extracts. It is correlated with considerable alkalinity of the midgut contents, which is characteristic of the Lepidoptera (Waterhouse 1949). The pH optima of the other insect proteinases serve to indicate their relationship to trypsin and their differences from cathepsin (note the comparisons made by Balls and Kies 1946).

TABLE 5
pH OPTIMA OF INSECT PROTEINASES

Insect	Reference	Substrate	pH Optimum	pH Optimum, Gelatin Viscosity Method (this paper)
<i>Tineola</i> larvae	Linderstrom-Lang and Duspiva (1936)	Casein, 2 hr. 40°C.	9.3	9.8
<i>Lucilia</i> larvae	Hobson (1931 <i>b</i>)	Collagen, 48 hr. 37°C.	8.5	
<i>Lucilia</i> larvae	Hobson (1931 <i>a</i>)	Gelatin, 24 hr. 37°C.	7.6	
<i>Periplaneta</i>	Wigglesworth (1928)	Gelatin, 24 hr. 37°C.	7.7	8.4
<i>Periplaneta</i>	Wigglesworth (1928)	Casein, 24 hr. 37°C.	7.1	
<i>Periplaneta</i>	Wigglesworth (1928)	Edestin, 24 hr. 37°C.	8.1	

(c) Oxidation-Reduction Potential

Most digestive proteases function in a medium in which the redox potential does not differ greatly from the range +100 to -100 mv. The observation of Linderstrom-Lang and Duspiva (1936), confirmed in the present investigation, that the oxidation-reduction potential of the midgut of the *Tineola* larva approximates -300 mv. suggested that the protease from this species may be more efficient at this potential. This hypothesis was strengthened by the results of Reiss and Achard (1943) who claimed that the tissue proteinase from *Bombyx* larvae was, in fact, sensitive to changes in oxidation-reduction potential, and further that its maximum hydrolytic effect occurred at about this same figure of -300 mv. The slight effect on *Tineola* proteinase of variations in oxidation-reduction potential over the physiological range indicates that in this particular *Tineola* proteinase is certainly not adapted to the conditions of oxidation-reduction potential under which it functions. A number of authors (Nagai 1939; Sizer 1945; Grob 1949) have reported effects of oxidizing and reducing agents on tryptic proteinases. These experiments have been carried out under a variety of conditions and by different methods and it is not possible to compare directly the results with those reported in this paper. The problems of finding suitable techniques for the study of the effects of differences in oxidation-reduction potential on proteinases have been stressed in Section II above. We have found the method employed in our experiments to be relatively free from errors and our results show no significant influence whatever of the effect of oxidation-reduction potential on the activity of the digestive proteinase from

Periplaneta and *Musca* and relatively small effects on the enzymes from the other insects examined. The effects on papain and trypsin are in the direction expected on the basis of previous work (Sizer 1945; Street 1949).

(d) Enzyme Activity

During the course of the work reported above, extensive observations have been made on two further aspects of *Tineola* proteinase that conflict with the views of Linderstrom-Lang and Duspiva (1936). These authors report a straight line relationship between enzyme concentration and activity, and this observation was repeated by Duspiva (1936). Some dozens of dilution curves of *Tineola* proteinase have been examined, over a period of 12 months, by a variety of methods for estimating proteinase activity, and in every case a typical Michaelis-Menten dilution curve has been obtained. No explanation can at present be offered for Linderstrom-Lang's finding, but since it does not conform to the theoretical dilution curve, attempts to repeat the result would be of interest.

Again, Linderstrom-Lang and Duspiva (1936) report that the preparations of *Tineola* proteinase were of very considerable activity. In fact, they state that acetone-dried preparations of *Tineola* midgut approach the proteolytic activity of Pancreatin "Merck." Table I will indicate that our preparations were far less active. Preparations made from different instars of *Tineola* larvae and by different methods did not possess activities approaching those reported by Linderstrom-Lang and Duspiva (1936). The considerable activity of acetone-dried caeca of *Locusta*, however, is noteworthy.

The considerations of the previous paragraphs indicate the similarity of the insect enzymes studied, and suggest that the differences observed between them are all of a minor character. It is therefore apparent that the proteinase from *Tineola* is in many respects not especially adapted to its unusual substrate and that the peculiarities of digestion of this species reside in factors other than its protein-digesting enzymes.

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VI. REFERENCES

- BALLS, A. K., and HOOVER, S. R. (1937).—The milk clotting action of papain. *J. Biol. Chem.* **121**: 737-45.
- BALLS, A. K., and KIES, M. W. (1946).—Proteases in "Enzymes and their Role in Wheat Technology," pp. 231-73. Ed. J. A. Anderson. (Interscience Publishers Inc.: New York.)
- COLLIER, H. B. (1941).—A trypsin-inhibiting fraction of *Ascaris*. *Canad. J. Res. B* **19**: 91-8.
- DAY, M. F., and POWNING, R. F. (1949).—A study of the processes of digestion in certain insects. *Aust. J. Sci. Res. B* **2** (2): 175-215.
- DUSPIVA, F. (1936).—Beiträge zur enzymatischen Histochemie. XXI. Die proteolytischen Enzyme der Kleider und Wachsmottenraupen. *Hoppe-Seyl Z.* **241**: 177-201.

- FRAENKEL-CONRAT, H., BEAN, R. S., and LINEWEAVER, H. (1949).—Essential groups for the interaction of ovomucoid (egg white trypsin inhibitor) and trypsin, and for tryptic activity. *J. Biol. Chem.* **177**: 385-403.
- GROB, D. (1949).—Proteolytic enzymes. III. Further studies on protein, polypeptide, and other inhibitors of serum proteinase, leucoproteinase, trypsin and papain. *J. Gen. Physiol.* **33** (2): 103-24.
- HAM, W. E., and SANDSTEDT, A. M. (1944).—A proteolytic inhibiting substance in the extract from unheated soybean meal. *J. Biol. Chem.* **154**: 505-6.
- HANKE, W. E., and KATZ, J. I. (1943).—An electrolytic method for controlling oxidation-reduction potential and its application in the study of anaerobiosis. *Arch. Biochem.* **2**: 183-200.
- HAWK, P. B., OSER, B. L., and SUMMERSON, W. H. (1947).—"Practical Physiological Chemistry," 12th Ed. (The Blakiston Company: Philadelphia.)
- HOBSON, R. P. (1931a).—Studies on the nutrition of blow-fly larvae. I. Structure and function of the alimentary tract. *J. Exp. Biol.* **8**: 109-23.
- HOBSON, R. P. (1931b).—An enzyme from blow-fly larvae (*Lucilia sericata*) which digests collagen in alkaline solution. *Biochem. J.* **25**: 1458-63.
- LENNOX, F. G. (1943).—The gelatin viscosity reduction method of measuring proteolytic activity. *J. Coun. Sci. Industr. Res. Aust.* **16**: 155-66.
- LINDERSTROM-LANG, K., and DUSPIVA, F. (1936).—Studies on enzymatic histochemistry. XVI. The digestion of keratin by the larvae of the clothes moth (*Tineola biselliella* Humm.). *C.R. Lab. Carlsberg* **21**: 53-83.
- LINEWEAVER, H., and MURRAY, C. W. (1947).—Identification of the trypsin inhibitor of egg white with ovomucoid. *J. Biol. Chem.* **171**: 565-81.
- NAGAI, Z. (1939).—Relation between oxidoreduction and tryptic and ereptic activities. *J. Biochem., Tokyo* **30**: 225-34.
- REISS, P., and ACHARD, G. (1943).—Influence du pH et du potentiel d'oxydation-reduction du milieu sur l'activité in vitro des protéinases tissulaires du Ver-à-soie. *Arch. Phys. Biol.* **17**: 88-90.
- SCHLOTTKE, E. (1937a).—Untersuchungen über die Verdauungsfermente von Insekten. 1. Die Verteilung der Fermente in Darmkanal von fleischfressenden Carabider und die Änderungen ihrer Konzentration während der Verdauung. *Z. vergl. Physiol.* **24**: 210-47.
- SCHLOTTKE, E. (1937b).—Die Abhängigkeit der Fermentgehaltes von der Art der Nahrung. Versuche an *Periplaneta orientalis* L. *Z. vergl. Physiol.* **24**: 463-82.
- SIZER, I. W. (1945).—Essential groups of crystalline chymotrypsin. *J. Biol. Chem.* **160**: 547-54.
- STREET, H. E. (1949).—Nitrogen metabolism of higher plants. *Adv. Enzymol.* **9**: 391-454.
- WAKSMAN, S. A., and DAVISON, W. C. (1926).—"Enzymes. Properties, Distribution, Methods & Applications." (Williams & Wilkins Co.: Baltimore.)
- WATERHOUSE, D. F. (1949).—The hydrogen ion concentration in the alimentary canal of larval and adult Lepidoptera. *Aust. J. Sci. Res. B* **2** (4): 428-37.
- WIGGLESWORTH, V. B. (1928).—Digestion in the cockroach. III. The digestion of proteins and fats. *Biochem. J.* **22**: 150-61.
- WIGGLESWORTH, V. B. (1939).—"The Principles of Insect Physiology." (Methuen & Co.: London.)

STUDIES ON THE DIGESTION OF WOOL BY INSECTS

III. A COMPARISON BETWEEN THE TRACHEATION OF THE MIDGUT OF *TINEOLA* LARVAE AND THAT OF OTHER INSECT TISSUES

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Summary

A study of the tracheation of nine tissues and organs of five species of insects reveals that the arrangement of tracheae and especially of tracheoles is determined by the structure of the tissue supplied. Adaptations are found in the ovary permitting the supply of the oocyte during its rapid enlargement. There is a correlation between the abundance of the tracheal supply of a tissue and its probable oxygen requirements.

The tracheal supply of the *Tineola* larval midgut is less well developed than that of many other insects, but a number of insects have an even less well developed supply. The poor tracheal supply of the midgut of *Tineola* larvae probably contributes to their ability to digest keratin, but other insects, which do not digest keratin, also have poor tracheation of the midgut.

Tracheolar anastomoses could not be found in any insect examined, although anastomoses of tracheae are frequent in some organs. Tracheal end cells are found in most organs and tissues, but differ in form in different tissues and in the same tissue in different species. They are absent from the crop and midgut caeca of *Periplaneta* and from wing muscles. Tracheae in the *Periplaneta* wing do not respond to injury and are fairly static. Nor is there proliferation of surrounding tracheae in a detracheated area.

I. INTRODUCTION

Very low oxidation-reduction potentials are maintained in the alimentary tract of certain insects, for example in the midgut contents of larvae of the clothes moth, *Tineola*, where the potential approximates -0.30 volts (Linderstrom-Lang and Duspiva 1936). The mechanism of the maintenance of this low potential has never been explained.

Uric acid forms a high proportion of *Tineola* faeces and, in view of the results of Leifert (1935) on *Antheraea* larvae, it seems likely that some of it must be produced from hypoxanthine by xanthine oxidase. This hypothesis is confirmed by qualitative tests which reveal the presence of xanthine oxidase in the gut of *Tineola*. The hypoxanthine-uric acid reaction has one of the lowest redox potentials recorded in a biological system (Green 1934), and may well be a factor contributing to the maintenance of the gut potential. Now, xanthine oxidase is inhibited by oxygen (Stadie and Hangaard 1945), which suggests that, if this enzyme is important, the tracheation of the midgut must be such as to restrict the supply of oxygen.

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A preliminary study of the tracheation of the midgut of larval *Tineola* showed that it was far less highly developed than that of *Blattella* (cf. Day and Powning 1949). It seemed desirable, therefore, to extend the comparison to other insects. With the exception of certain organs like the silk gland, surprisingly little collated information about the tracheation of insect tissues is to be found in the literature. Those reports which do exist deal mainly with the possible intracellular nature of tracheoles (see Keister 1948), the presence of liquid in the tracheoles (see Wigglesworth 1931), and the existence of tracheolar anastomoses.

It was apparent that further comparative anatomical information was required, and some data on this subject are presented in this paper. These anatomical considerations suggested related problems of tracheation and the data reported have provided some information on the function of the tracheal system.

It seemed likely that a thorough study of many tissues of a few species would provide useful preliminary data, and so nine organs (heart, nerve, foregut, midgut, hindgut, rectum, malpighian tubules, fat body, and muscle (wing and body muscle, when possible)) were studied in five species, namely *Ctenolepisma longicaudata* Esch., *Periplaneta americana* (L.), larvae and adults of *Tenebrio molitor* L., larvae of *Tineola bisselliella* Humm., and larvae and adults of *Lucilia cuprina* Wied. Other species used in later comparisons will be referred to below. All species were terrestrial and none would be expected to have a peculiar tracheal system correlated with an unusual habitat.

II. METHODS

This work necessitated a detailed investigation of the relative effectiveness of several methods for making the tracheoles visible. It was found that no one method was satisfactory for all species, and all of the following methods were finally used with each species:

(1) Dark field observation of spreads of living tissue mounted in Toisson's solution. This was useful for all species, especially for larvae of *Lucilia*, which could not be studied by methods (3) and (4) below. Toisson's solution has the following composition: glycerine 30 ml., sodium sulphate 8 g., sodium chloride 1 g., methyl violet 0.025 g., distilled water 160 ml.

(2) Observation with the phase contrast microscope. Cooke, Troughton, and Simms phase contrast equipment was employed, and excellent differentiation of tracheoles was observed at magnifications up to 1425x. Both this and method (1) required the use of thin spreads, a condition not easily met in all tissues, especially of the larger species.

(3) Trypan blue injections by the method of Hagmann (1940) had the advantage over methods (1) and (2) of permanence. Spreads, rather than sections, provided the most generally useful preparations.

(4) The osmic acid method for studying tracheal end cells. This very useful method consisted of suspending the insects in a small cage over a 2 per cent. solution of osmic acid for periods of 1 to 4 hours, depending on the species,

washing in water, dehydrating, and clearing, followed by the preparation of spreads. Sections of osmic acid-impregnated material were also used but were less useful than the tissue spreads.

(5) The silver nitrate method as used for the demonstration of Buck's (1948) "possible ultratracheolar network." Buck (personal communication) has described this method as follows: "Soak the fresh tissue in pieces of about 1 to 2 mm.³ in 1 per cent. silver nitrate overnight in the dark, then remove tissue to a drop of glycerine on a slide, tease out, and place in the sunlight."

(6) Acetic orcein, on fresh or Carnoy-fixed material. This was useful for studying the tracheal epithelium.

Many attempts were made to use the osmic acid and trypan blue injection methods on *Lucilia* larvae. The osmic acid was administered under vacuum, under CO₂ or ether anaesthesia, and after the anterior and posterior spiracular closing mechanism had been destroyed; and the injections were attempted on anaesthetized larvae, larvae killed by hot water, and larvae with their spiracular closing mechanisms punctured. None was successful. Nerves were stained by the methylene blue method outlined by Day and Powning (1949).

Photomicrographs or scale drawings were made of all preparations for ease of comparison.

III. OBSERVATIONS

(a) *Arrangement of Tracheae in Tissues*

A comparison between similar tissues of different species shows clearly that the arrangement of tracheae in tissues, though typically arborescent, is determined by the fine structure of the tissue supplied. Thus, the tracheation of abdominal skeletal muscles in all species studied is characterized by main trunks with which the branches make approximate right angles and from these many fine branches run parallel with the muscle fibres. Similarly, the tracheation of ganglia of the ventral nerve cord follows a very regular pattern in all species (Plate 1, Fig. 1). Large trunks enter the ganglion from each side, branch fairly regularly and send long, fine branches down the length of the nerve cord. Again, the distribution of tracheae in the fat body is characteristic in all species studied (Plate 1, Fig. 2). Large trunks run among the large fat body cells, branching infrequently; branches usually form an acute angle between them and finer branches are given off to individual cells.

It is clear that the statement sometimes still quoted in textbooks that every cell in the body of an insect is tracheolated is inaccurate. Every cell of certain tissues, e.g. muscle, may be tracheolated, but many epithelial cells are well removed from the nearest tracheole. Keister (1948) mentions that the digestive tract and certain other organs of *Sciara* larvae are never tracheated.

The arrangement of tracheae in tissues is always such as to permit flexion and considerable movement of viscera. In muscle the finer branches are very sinuous, especially when the muscle is in the contracted condition. Modifications of tracheal patterns are encountered infrequently. An interesting example

is found in the insect ovary, where there is an unusual degree of tracheal folding on the surface of small oocytes (Plate 1, Fig. 3). This permits tracheae to supply rapidly developing oocytes without extensive growth.

(b) *Relative Abundance of Tracheae in Tissues*

Even though tissue spreads are not of uniform thickness, observations at a restricted focal plane under high magnification permit a close approximation to the "quantitative anatomy" which Krogh (1929) considers essential to the proper understanding of the details of physical respiration. Spreads are much superior to sections for such observations. In general, trypan blue injections showed more and finer tracheoles than the other methods. A comparison of many such preparations demonstrates a relationship between the degree of tracheation of organs and their probable oxygen requirements. Until the metabolism of various insect tissues has been studied such comparisons must be tentative, but the relationship is shown clearly in a comparison of, for example, wing muscle, abdominal skeletal muscle, brain, and fat body. The only organ with a high metabolic rate (as indicated by its tracheation) that might not have been expected is the rectum, but rectal pads are well known to be unusually richly tracheated in all species in which they have been examined. The malpighian tubules, normally poorly tracheated, are also well supplied with tracheae where they are associated with the rectum.

There are in most species differences in the degree of tracheation in different regions of the midgut, but the significance of such variations is not known. The midgut of *Tineola* larvae is better tracheated at its anterior end than at its posterior end, but is well supplied with tracheae throughout its length (Plate 1, Fig. 4, and Plate 2, Fig. 11). A comparison between the tracheation of several larval Microlepidoptera (*Gnorimoschema* (Plate 1, Fig. 5), *Sitotroga*, *Ephestia*, and *Plutella* (Plate 1, Fig. 6)) has shown that that of *Tineola* is less well developed than those of the other species of comparable size. In the anthelid, *Pterolocera amplicornis* Wlk., which is a much larger species whose mature larvae weigh about 0.7 g., almost every midgut cell is tracheated. The midgut is of the order of 0.5 cm. in diameter, so that thorough tracheation would be necessary to supply oxygen to the centre of the food mass. Yet the tracheal pattern is quite comparable to that of *Tineola* and there are no anastomoses as are found in *Blattella* (Day and Powning 1949) or *Periplaneta* (Plate 2, Fig. 7). Nor do tracheal vesicles occur, such as Metalnikov (1908) and G bler (1936) have described in larvae of *Galleria mellonella*. There is, at present, no reasonable explanation for the unusually rich tracheation of the midgut of *Galleria*.

It has been demonstrated from these comparisons that the tracheation of the larval midgut of *Tineola* is less well developed than in most other insects (Plate 2, Figs. 7 and 9) and in some other lepidopterous species of comparable size (Plate 1, Figs. 4, 5, and 6). However, it is better developed than that of many other species also examined, for example *Anthrenus* and *Attagenus* larvae (cf. also *Sciara* larva (Keister 1948)).

(c) *Tracheal End Cells*

The most significant feature of a tracheal system is its ability to transfer oxygen to tissues in the body. The sites of transfer are considered to be demonstrated by the reduction of osmic acid to the metal, which produces a darkening at the site of transfer. This has been known for 75 years (see Wigglesworth 1931), but no comparative study of the sites of osmic acid reduction is available. Study of the tracheal end cells in nine organs of *Periplaneta*, larval and adult *Tenebrio*, larval *Tineola*, and adult *Lucilia* resulted in the following conclusions:

(i) Osmic acid is not normally reduced in the walls of large tracheal trunks or in tracheal branches of most insects (Plate 2, Figs. 8 and 10).

(ii) The microlepidopterous larvae (Plate 2, Fig. 12) examined and *Ctenolepisma* constitute an exception to this, however, and blackening of tracheal trunks usually occurs in these species right up to the spiracles.

(iii) Wing muscle is also exceptional. The entire muscle is darkened during exposure to osmic acid.

(iv) In the majority of tissues, reduction occurs at well-defined regions along the trachea or tracheole, frequently at the tracheal end cell. The latter has been observed in almost all tissues, except wing muscles and the crop or midgut caeca of *Periplaneta*.

(v) Tracheal end cells differ considerably in form in different tissues, and in the same tissue in different species. Frequently observed types are shown in Plate 2, Figures 8, 10, and 12. Sometimes the unstained tracheole can be discerned distal to the osmophile region. This does not seem to be due to the fact that this section is normally fluid-filled, for two reasons:

(a) The position of the osmophile region does not change if the insect is treated so that the amount of fluid in the tracheoles is reduced (e.g. exposure to CO₂) immediately before or during the exposure to osmic acid;

(b) Instances have been found of two separated osmophile regions along the length of a single tracheole.

(vi) Within a species there appears to be a relationship between the oxygen requirements of a tissue and its supply of tracheal end cells. Thus, in *Periplaneta* the order of decreasing abundance of tracheal end cells is roughly: muscle, rectum, midgut, hindgut, nerve, heart, malpighian tubules, and fat body. A comparison between the tracheal end cells of the *Tineola* larval midgut and those of the other species examined shows that they are well developed in *Tineola* but fewer in number, supporting the hypothesis that the oxygen supply to the midgut of *Tineola* is less abundant than that of many other insects of comparable size.

(d) *The Silver Nitrate "Network"*

Cajal (1890) observed, with the Golgi technique, a network binding tracheoles together in insect muscles, and Buck (1948) has observed with silver

nitrate what he tentatively describes as an ultratracheolar network in the light organ of lampyrids. It has been found that many tissues show characteristic patterns of deposited silver when treated by the method described above. Thus, muscles show a beautiful pattern of granules, frequently paired, regularly arranged both longitudinally along the fibril and transversely across the striations. The details of the pattern vary from muscle to muscle and from species to species. The relation of the granules to the striations has not been determined, but it seems certain that they represent silver deposits related to the fine structure of the muscle fibre. They do not appear to be connected with the numerous tracheoles which ramify between the fibrils.

On the surface of the male accessory glands of *Periplaneta* a complex network of fine granules outlines the cell boundaries, whereas the cells of the malpighian tubules are covered uniformly with granules and the cell walls are not outlined by them. The same is true of the salivary glands of larval *Lucilia*.

On the surface of the larval *Tineola* midgut an irregular network of deposited silver appears to be related to the tracheoles, but it is much coarser than that found by Buck (1948) on the cells of the light organ.

Summarizing these and similar observations on many tissues of *Periplaneta*, *Tenebrio* larvae and adults, *Tineola* larvae, and *Lucilia* larvae and adults, the generalization seems warranted that silver granules are deposited on tissues in a manner dependent upon the fine surface structure of the tissue; but no evidence was obtained to substantiate the existence of an ultratracheolar network in any of the tissues examined.

(e) *Histological Structure of Tracheae and Tracheoles*

Tracheae are remarkably uniform in structure along their length. Careful study of acetic orcein preparations has failed to reveal any structures which might be involved in constricting the lumen. There is apparently no "physiological reserve" as there is of arterioles and capillaries of vertebrates. Similarly, there are in the species studied no valvular mechanisms providing a unidirectional flow, or any internal mechanism for increasing the intratracheal pressure, although this can be accomplished by spiracular and body movements (McCutcheon 1940). A peculiar intratracheal valve has been described by Webb (1945) in *Melophagus*, demonstrating that the above generalizations are not without exceptions among insects.

Tracheae are not innervated. This was shown by a detailed examination of methylene blue preparations of the tissues of *Periplaneta* by the method of Kuwana as outlined by Day and Powning (1949). Many nerves in many organs were traced, but none was ever observed to terminate either on a large tracheal trunk or at a tracheolar ending. Frequently, nerves run along side tracheal trunks and from the latter small tracheae or tracheoles run to the nerve at irregular intervals. Frequently the nerves and tracheae branch together, but the tracheae become smaller and end, whereas the nerve may continue undiminished in diameter. The best demonstration of the relationship between the

two systems is seen in the rectum, which is both well tracheated and well innervated. It is clear that the nervous system of this organ is tracheated but that the tracheal system is not innervated. The number of fine nerve branches is only a small fraction of the number of tracheoles.

It has been claimed that the "dark staining sheath" of Dahlgren (1917), which surrounds the tracheoles, provides a mechanism for controlling the oxygen supply to tissues, especially to the light organ of fire flies. Such control could only be exercised over the whole organ by a nervous or a humoral mechanism, and neither seems likely. Available data indicate that control of the tracheal system occurs only at the spiracles (the muscles of which are, of course, innervated) and at the tracheoles by movements of their fluid contents. Indirect control is also produced by the movements of ventilation.

The fine structure of the tracheal epithelium was studied by the acetic-orcein technique. The general belief that the tracheal epithelium of different insects is not markedly different is without foundation. It has been found that:

(1) The tracheal epithelium of the larvae studied is more conspicuous than that of their respective adults.

(2) The epithelium of the smaller trunks is relatively thicker than that of the larger trunks.

(3) Mitoses are not infrequent in the tracheal epithelia of larvae, and aberrant nuclei are frequently found in both larvae and adults.

(4) Cell walls vary in conspicuousness, but could always be demonstrated by appropriate techniques, methylene blue being useful for this purpose.

The observations made in the course of this work suggest, as certainly as possible by the use of light microscopy alone, that, in the majority of insect tissues, the tracheoles end blindly without anastomoses. This is in contradiction to the opinion of Snodgrass (1935, p. 450) who says that in tissues that have been studied the ultimate branches of the tracheoles "have been found to anastomose in a fine capillary network over the tissue cells" In the light organ of lampyrids, which is particularly thoroughly tracheated, anastomosing tracheoles have been described by many authors and may occur (Buck, personal communication), even though electron microscopy has shown that many tracheoles end blindly (Buck 1948). In view of the specialization of the light organ the presence of anastomoses in that tissue would not invalidate the generalization that they do not ordinarily occur (cf. also Keister 1948; Richards and Korda 1950). Tracheal anastomoses, on the other hand, may be found not infrequently in tissues that are well supplied with tracheae, as for example the midgut of *Periplaneta* or *Blattella*.

(f) *Static Nature of Tracheae*

An organ implanted into a larval insect becomes tracheated as does a normal organ of the host (Meisenheimer 1907). The mechanism by which this occurs has not been investigated. In vertebrates, the blood vessels are broken down and rebuilt continually. Although such dynamic changes are not characteristic of insect tracheae, the occurrence of mitoses in the epithelium

suggested that some growth of tracheae may go on in the adult insect. An attempt was made to study tracheal changes in the cockroach wing after wounding. Cuts were made in the tegumen, and drawings of the surrounding regions were made immediately and at intervals of about a week thereafter for six weeks. In some insects the tracheae distal to the cuts became invisible within one week, probably being filled with liquid. No other changes were visible. In other specimens practically no change could be seen in the tracheae even after six weeks. Special attention was paid to possible changes in fine branches proximal to the cut and to the adjacent intact tracheae to determine any growth or anastomoses. No such changes were observed.

These observations indicate that the tracheae in the adult have lost the capacity to repair damage to an area deprived of its normal oxygen supply, and that, as far as can be observed at the magnifications that could be employed (100x), tracheae in the *Periplaneta* tegumen do not have the ability to undergo structural alteration once the insect is adult. If it is objected that the tegumen is not a favourable site for such a study because of the probable ease of aeration even without tracheae, corroborative evidence for the static nature of tracheae can be found in the midgut of termites (*Coptotermes*, *Nasutitermes*). In workers the midgut is relatively poorly tracheated, but that of the comparably sized alates is extraordinarily well developed. In the event of these alates becoming physogastric, growth of the alimentary canal can occur and it can be adequately tracheated without extensive changes in the tracheae already present.

(g) Effects of Pressure Changes

The above observations showed the static nature of the tracheae in the cockroach tegumen and suggested a study of their reaction to decreases and increases in environmental pressure. *Periplaneta* adults were lightly anaesthetized with carbon dioxide and fixed by plasticine in a clear plastic chamber in which the pressure could be increased or decreased. A tegumen was held flat against the upper surface by a coverslip held in position by stopcock grease. Changes in tegumen tracheae could be observed with magnifications of 100x. Higher magnifications could not be used because of the technical difficulty of obtaining a window thin enough but still strong enough.

The tracheae are practically unaffected by a vacuum of 8 in. of mercury. At 27 in. of mercury they collapse in some specimens, particularly those that have recently moulted. In others, however, even at this pressure they are practically unaffected. On sudden return to atmospheric pressure they regain their normal shape within a few seconds and the insect is quite unaffected. This can be repeated many times with similar results. Since this pressure is equivalent to a height of about 50,000 feet above sea level it is readily appreciated that the high altitudes at which insects have been taken are unlikely to affect their physical respiration adversely.

At increased pressures of 4 lb. per sq. in. (equivalent to a depth of only about 8 ft. under water) the tracheae collapse, but regain their normal condi-

tion when the pressure is released. Again the insects suffer only very temporarily from the effects, and can undergo repeated compressions and sudden decompressions without apparent physiological embarrassment.

IV. DISCUSSION

Leaving aside special adaptations for parasitic and aquatic respiration, and respiration of unusual types, the tracheal system is probably the simplest system, anatomically and histologically, in insects. This simplicity is correlated with its uncomplicated function. Thus, the system responsible for gas transport (including the gases of respiration) in vertebrates also functions in the transport of food materials and hormones, in temperature control, and in the maintenance of the internal environment of the organism; the tracheal system of insects functions only in the transport of gases. In vertebrates the "arrangement of the capillaries is determined by the architecture of the particular organ or tissue whose life lines they constitute" (Cowdry 1938, p. 121), and this is similarly evident for the small tracheae and tracheoles. The similarity between the tracheation of an insect muscle and the capillaries of a vertebrate (e.g. Fig. 1 of Krogh 1929) is most striking. But, although the arrangement of tracheae in tissues is dependent upon the tissue supplied, the abundance of the tracheation seems to be dependent upon the oxygen requirements of that tissue.

All detailed anatomical studies, except that of Webb (1945), have failed to demonstrate any complexities in the structure of the tracheae. They provide a pathway for gaseous diffusion, a function generally requiring no active participation of the organ system. Correlated with its single function the organ system is one of unusual simplicity. There is no evidence that, when adequate ventilation occurs, the tracheal system is, in any respect, limiting in the development of increased size. Similarly, the tracheal system is suitable for physical respiration in an unusual variety of terrestrial habitats.

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VI. REFERENCES

- BUCK, J. B. (1948).—The anatomy and physiology of the light organ in fireflies. *Ann. N.Y. Acad. Sci.* 49: 397-482.
- CAJAL, S. R. Y (1890).—Coloration par la méthode de Golgi des trachées et des nerfs dans les muscles des ailes des insectes. *Z. wiss. Mikr.* 7: 332-42.
- COWDRY, E. (1938).—"Textbook of Histology." 2nd Ed. (Lea and Febiger: Philadelphia.)
- DAHLGREN, U. (1917).—The production of light by animals. The luminous insects. *J. Franklin Inst.* 183: 79-94, 211-20, 323-48, 593-624.
- DAY, M. F., and POWNING, R. F. (1949).—A study of the processes of digestion in certain insects. *Aust. J. Sci. Res. B* 2: 175-215.

- GÄBLER, H. (1936).—Beitrag zur Kenntnis des Tracheensystems der Raupen von *Achroea gresilla* F. und *Galleria mellonella* L. *Zool. Anz.* **116**: 176-85.
- GREEN, D. E. (1934).—Studies on reversible dehydrogenase systems. II. The reversibility of the xanthine oxidase system. *Biochem. J.* **28**: 1550-60.
- HAGMANN, L. E. (1940).—A method for injecting insect tracheae permanently. *Stain Tech.* **15**: 115-8.
- KEISTER, M. L. (1948).—The morphogenesis of the tracheal system of *Sciara*. *J. Morph.* **83**: 373-424.
- KROGH, A. (1929).—"The Anatomy and Physiology of the Capillaries." (Yale Univ. Press: New Haven.)
- LEIFERT, H. (1935).—Untersuchungen über den Exkretstoffwechsel bei Eiern, Raupen und Puppen von *Antheraea pernyi*. *Zool. Jber.* **55**: 131-90.
- LINDERSTROM-LANG, K., and DUSPIVA, F. (1936).—Studies on enzymatic histochemistry. XVI. The digestion of keratin by the larvae of the clothes moth (*Tineola bisselliella* (Humm.). *C.R. Lab. Carlsberg* **21**: 53-83.
- MCCUTCHEON, F. H. (1940).—The respiratory mechanism of the grasshopper. *Ann. Ent. Soc. Amer.* **33**: 33-55.
- MEISENHEIMER, J. (1907).—Ergbnisse einiger Versuchsreihen über Extirpation und Transplantation der Geschlechtsdrüsen bei Schmetterlingen. *Zool. Anz.* **32**: 393-400.
- METALNIKOV, S. (1908).—Recherches expérimentales sur les chenilles de *Galleria mellonella*. *Arch. Zool. Exp. Gén.* (4) **8**: 489-588.
- RICHARDS, A. G., and KORDA, F. H. (1950).—Studies on arthropod cuticle. IV. An electron microscope survey of the intima of arthropod tracheae. *Ann. Ent. Soc. Amer.* **43** (1): 49-71.
- SNODGRASS, R. E. (1935).—"The Principles of Insect Morphology." (McGraw-Hill Book Co. Inc.: New York.)
- STADIE, W. C., and HANGAARD, N. (1945).—Oxygen poisoning. VII. The effect of high oxygen pressure on enzymes; uricase, xanthine oxidase and d-amino acid oxidase. *J. Biol. Chem.* **161**: 181-8.
- WEBB, J. E. (1945).—On the respiratory mechanism of *Melophagus ovinus* L. (Dipt.). *Proc. Zool. Soc.* **115**: 218-50.
- WIGGLESWORTH, V. B. (1931).—The respiration of insects. *Biol. Rev.* **6**: 181-220.

EXPLANATION OF PLATES 1 AND 2

PLATE 1

Tracheation of insect tissues

- Fig. 1.—*Periplaneta* abdominal ganglion, trypan blue injection, showing type of tracheation in nervous tissue.
- Fig. 2.—*Periplaneta* fat body, phase contrast spread, showing characteristic type of tracheal distribution and branching in this tissue.
- Fig. 3.—*Tenebrio* adult ovary, trypan blue injection, showing sinuous tracheae on small oocytes, and straighter tracheae on larger oocytes.
- Figs. 4, 5, and 6.—Spreads of midgut under dark field of *Gnorimoschema*, *Plutella*, and *Tineola*, respectively, illustrating comparative tracheation.

PLATE 2

Tracheation of insect midgut

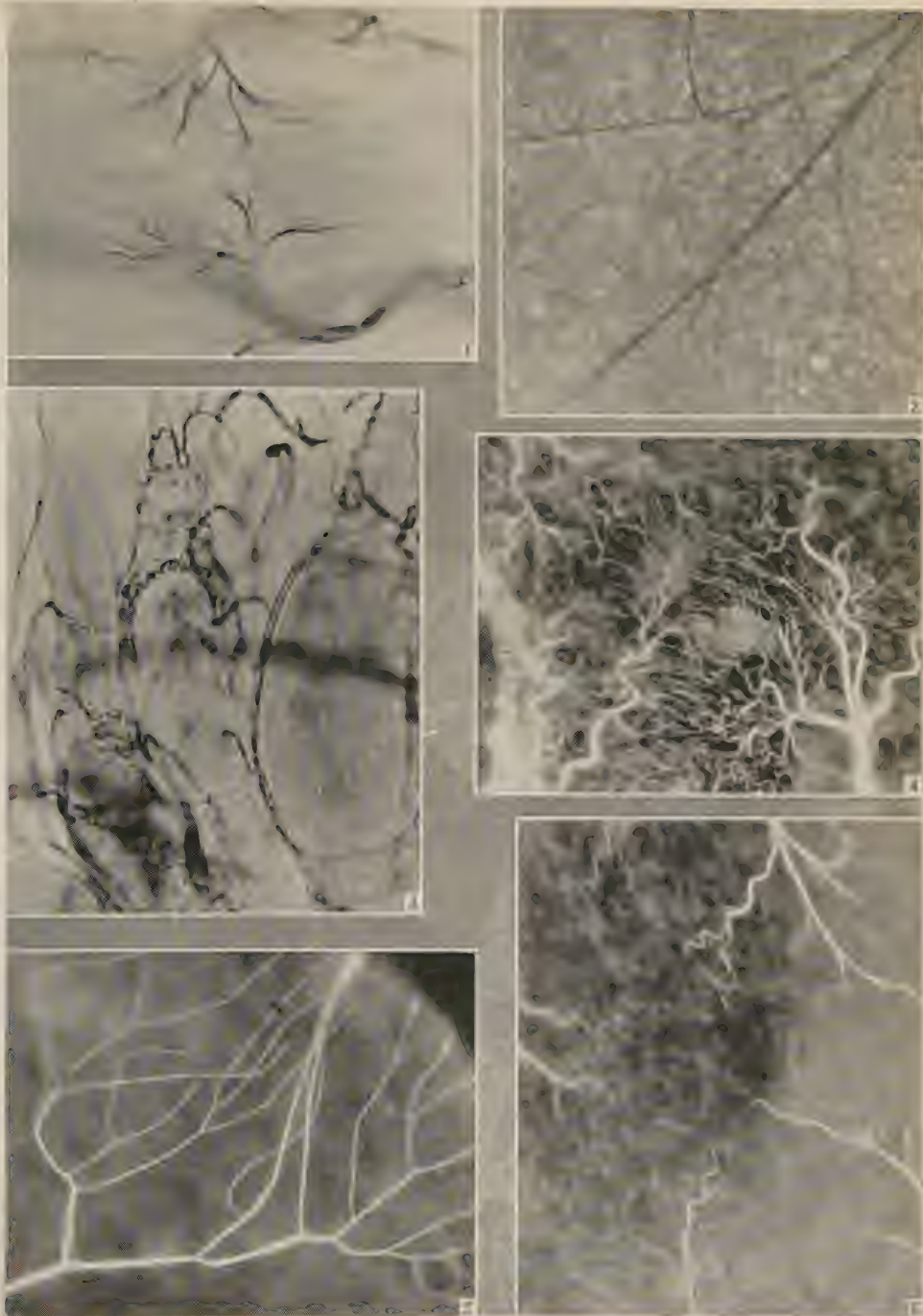
Figures 7, 9, and 11 with 8x ocular and 10x objective

Figures 8, 10, and 12 with 8x ocular and 33x objective

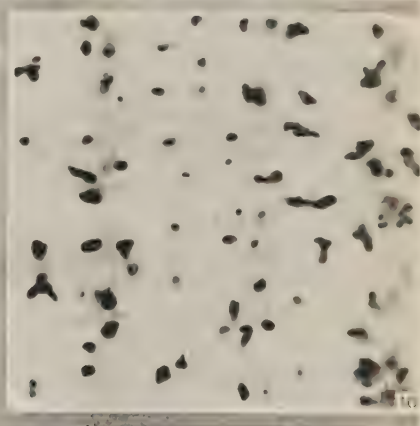
- Fig. 7.—*Periplaneta* midgut, spread of trypan blue injection, showing thorough tracheation and anastomosing tracheae.

- Fig. 8.—*Periplaneta* midgut, osmic acid preparation showing tracheal end cells surrounding every nidus.
- Fig. 9.—*Tenebrio* larval midgut, spread of trypan blue injection, showing thorough tracheation but absence of anastomoses.
- Fig. 10.—*Tenebrio* larval midgut, osmic acid preparation showing isolated tracheal end cells uniformly scattered over the muscularis.
- Fig. 11.—*Tineola* larval midgut showing relatively sparse tracheation.
- Fig. 12.—*Tineola* larval midgut, osmic acid preparation, showing characteristic form of tracheal end cells and staining of tracheoles and of connecting tracheae.

DIGESTION OF WOOL BY INSECTS. III



DIGESTION OF WOOL BY INSECTS. III



THE PHYSIOLOGY OF GROWTH IN APPLE FRUITS

I. CELL SIZE, CELL NUMBER, AND FRUIT DEVELOPMENT

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Summary

The problem of fruit size in the Australian apple variety Granny Smith was examined in relation to mean cell size and mean cell number. Cell size gradients in the fruit and changes in cell shape and packing during development were noted.

Observations of workers on other varieties that cell division ceased within four weeks of pollination were confirmed. Variation in size of fruits at maturity was shown to be due mostly to variation in cell number and only to a small extent to mean cell size. Cell enlargement was shown to continue throughout the life of the fruits on the tree.

I. INTRODUCTION

Growth of fruits is a problem, not only of considerable plant physiological interest but also of outstanding economic importance. In apples, for instance, fruit size has for a long time been regarded as an important factor in determining the keeping quality of apples in storage. Since keeping quality is related to the physiology of the fruit, it is of considerable interest to investigate the anatomical and histological causes of differences in fruit size and to relate these to physiological phenomena, both during the development of the fruit and during its senescent life after removal from the tree. The work described in this paper was undertaken to study the relationship between cell size and fruit size, and in a second paper this will be related to physiological and biochemical changes.

Smith (1940) has determined the cell size and cell number of several varieties of English apples. He has related size and number of cells in the flesh of mature apples to the sizes of the fruits at maturity, and correlated respiration rates and keeping quality with cell number. This work was based on mean cell size determinations made on tissue at a standard depth under the skin of the fruit. Smith suggested that if increase in size of the bigger apples were due entirely to more cells, then cell sizes of both large and small apples should be the same. If, on the other hand, increase in fruit weight were to be accounted for by cell enlargement alone, then cell size should have doubled for a doubling in apple weight. Since neither of these postulates was

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obeyed, he concluded that differences in sizes of mature apples were due both to the amount of cell division and to the degree of cell enlargement. Further, Smith has correlated the respiration rate of different varieties with cell number and has shown that there appears to be a correlation between respiration rate, expressed on a unit fresh weight basis, and number of cells per gram of tissue. It is also pointed out that those varieties with the greater numbers of cells and the higher respiration rates per unit weight are also those which have poorer keeping quality. In a more recent paper, Smith (1950) has extended these observations to evaluate the part played by cell multiplication and cell enlargement in the development of fruits of a number of varieties. In this paper, similar observations that have been made on the Australian variety Granny Smith, will be described.

Various authors have concerned themselves with the anatomy and histology of the apple fruit, but have not been primarily interested in the problem of size. Tetley (1930, 1931) studied the morphology and cytology of developing Bramley's Seedling apples and established the fact that cell division ceases a few weeks after fruit set. Thereafter, increase in size is mostly due to cell enlargement. MacDaniels (1940) was concerned with the morphology of pome fruits and MacArthur and Wetmore (1939, 1941) with developmental studies of the anatomy of McIntosh Red and Wagener apples. Apart from the work of Smith, the only investigation that bears directly on cell size is that of Tukey and Young (1942), who studied the varieties Lodi, Early Harvest, Twenty Ounce, McIntosh, and Rome, with histological observations on the McIntosh. Tukey and Young record that cell division in the pith seems to have ceased by three weeks after full blossom and, thereafter, increase in size is due to increase in the size of cells and intercellular spaces, some cells reaching $150 \times 300 \mu$. In the cortical region also, cell division appears complete three weeks after full bloom and thereafter cell enlargement is responsible for increase in size, some cells attaining a size of $197 \times 340 \mu$. Observations on the number of cells across the cortex indicate that there is very little increase in number after three weeks from full blossom.

The interpretation of the morphology of the apple fruit is uncertain. Two theories have been suggested. The receptacular theory considers the fruit to be composed of five drupe-like carpels contained in a fleshy receptacle, which is therefore regarded as a fleshy development of the stem, so that areas comparable with pith and cortex can be defined. The appendicular theory considers the carpels to be enclosed by fleshy tissue derived from the fused and enlarged bases of the floral appendages so that the apple flesh represents the floral tube formed from fused petals, sepals, and stamens. It is not necessary to discuss the relative merits of these two theories in this work; for convenience, the receptacular theory is followed and the terms pith and cortex are used for the regions respectively inside and outside the ring of ten vascular bundles.

This paper describes attempts to determine cell size, cell number, gradients in cell size within the fruit, and gross morphological changes during development, and to correlate fruit size, cell size, and cell number.

II. MATERIAL AND METHODS

The material used was the Granny Smith variety and was obtained from four trees. Tree 1 is at Orange, N.S.W., and trees 2, 3, and 4 are at the New South Wales Department of Agriculture Experiment Farm at Bathurst, N.S.W.

Two main experiments were carried out. For the first of these, *the experiment on size in mature fruits*, 43 fruits of weights ranging from 73.8 g. to 251 g. were taken from tree 1 at time of commercial picking in 1947, and the cell sizes determined. At commercial picking in 1948, another 24 fruits were taken, ranging in size from 101 g. to 210 g. and the cell sizes determined.

TABLE 1
DATES OF PICKING OF EARLY SAMPLES

Sample	Date	Days from Full Blossom
A	10.xi.47	21
B	17.xi.47	28
C	24.xi.47	35
D	9.xii.47	42
E	17.iii.48	149

For the second experiment, the *fruit growth experiment*, developing fruits were sampled at intervals from the Experiment Farm trees. Date of full blossom was taken as the approximate date of first petal fall, October 20, 1947. Between petal fall and December 10, 1947, samples were taken from tree 2 to examine the cell division stage. Fruit was not taken from the main experimental trees (3 and 4) over this period so as to avoid overthinning these trees. Fruit taken from tree 2 over this period consisted of four samples each of 24 fruits; sizes ranged from 1.4 to 20.95 g. Table 1 shows the dates of picking

TABLE 2
DATE OF PICKING AND DAYS FROM FULL BLOSSOM OF SUCCESSIVE SAMPLES
FROM TWO TREES

Pick	No. of Fruit		Date	Days from Full Blossom
	Tree 3	Tree 4		
1	20	—	10.xii.47	51
2	20	—	31.xii.47	72
3	10	10	4.ii.48	107
4	10	10	18.ii.48	121
5	10	10	3.iii.48	135
6	10	10	31.iii.48	163
7	10	10	14.iv.48	177
8	10	10	28.iv.48	191
9	9	10	12.v.48	205

of these samples and of another sample taken from the same tree much later. From December 10, 1947 onwards, fruit was taken from the main experimental trees as shown in Table 2. Commercial picking date was April 28, 1948 — 191 days from full blossom.

(a) *Fruit Size*.—Each fruit was weighed with the stalk attached and was then cut in half transversely. Tracing paper was placed on the cut surface of the half fruit, and the regions of the tissues outlined; the areas of the various tissues were determined from these tracings with a planimeter.

(b) *Fixing Material*.—Anatomical studies of apple tissue are difficult because of the large intercellular spaces, which are filled with air; all cell examinations must be made with fixed material from which air has been excluded. Sections of the tissue to be examined were cut and fixed in acetic-alcohol (1 : 3), which removed the air from the intercellular spaces. After fixing, sections were stored in 70 per cent. alcohol until required. When the fruits were being examined for cell division the fixative used was formalin-alcohol (6 ml. formalin in 100 ml. 70 per cent. alcohol). The method of fixation made negligible difference to the mean cell diameters. This was tested in two ways:

- (1) cylinders of tissue were measured when cut, and again after fixation; the largest change in dimensions was only about 3 per cent., and
- (2) sections were examined in isotonic solutions and then transferred to fixative; no significant change in mean cell diameter was detected after fixation.

(c) *Staining*.—When the tissue of young fruits was being examined for cell divisions, safranin-haematoxylin was used. Water blue stained the nuclei and the cytoplasm of cells in mature tissues. It was unnecessary to use any stain in the measurement of cell size as the cell outlines in apple tissue are very distinct.

(d) *Estimation of Cross-Sectional Dimensions of Cells*.—A micro-projector was found to be very satisfactory to trace cell outlines. A calibrated micrometer was used to obtain the magnification (approximately $\times 100$) of the projector.

Since the form of the cells was found to approximate to spheres or to oblate spheroids (elliptical on the major axis of rotation), the volume was calculated from the tracing of the cells by measuring the major and minor axes and using the formula $\frac{4}{3} \pi a b^2$ where a is half the length of the major axis and b is half the length of the minor axis.

(e) *Sampling*.—Sampling was done in different regions of the fruits to establish the gradients in cell size. Four regions of different cell size can be distinguished: the skin region of very small cells, the mid-cortical region in which the cells are much larger, the region of very small cells round the vascular tissue, and the pith region consisting of large cells, mostly elongated along the radius of the fruit. Although standard deviations were always high, indicating variability in cell size even within one region, it was found that measurement of 20 or 25 cells was sufficient for a sample of the mid cortex or pith, though in some experiments large numbers of cells were measured.

As the apple increases in size, the cells elongate along the radius of the fruit, so that cells that appear approximately circular in tangential longitudinal

section are oval in both radial longitudinal section and transverse section. Consequently, the measurements of the cells were taken in transverse section. In most determinations the sections were taken from the equatorial radii of the fruits.

III. RESULTS

(a) Size in Mature Fruits

(i) *Mean Cell Size.*—Measurements of 25 cells in the mid region of the cortex were made and the mean volume of cells was calculated. Mean cell volume is plotted against fruit weight in Figure 1. The cell size of fruits of the 1948 pick appears to be slightly less than that of the 1947 pick, but in each case there is little increase in cell volume with increase in weight. Thus, taking a line of best fit (drawn by inspection) to the 1947 sample, the mean cell volume of a 70 g. mature fruit is 0.0030 cu. mm. and that of a 250 g. mature fruit is 0.0046 cu. mm., i.e. as the tissue volume measured by weight (assuming that the specific gravity of the cells does not change appreciably) increases 3.6 times, the mean volume of the cortical cells increases only 1.5 times. This

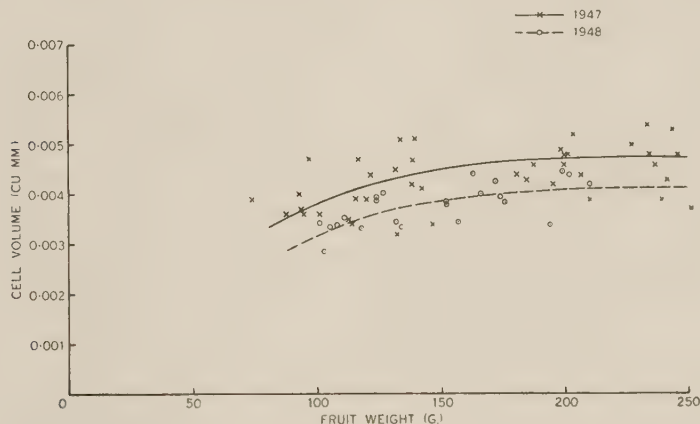


Fig. 1.—Relationship of mean volume of mid-cortical cells to fruit weight in apples from the same tree in two seasons.

is even more striking than the difference Smith (1940) obtained and interpreted as being due to a difference in cell number in the larger fruit. These results show that the difference in flesh volume with size of different mature fruits cannot be accounted for by the difference of the volumes of cells from the mid-cortical region. It could be accounted for by differences in cell number, provided that the mean cell size for the mid-cortical region can be taken as a reasonable value for the true mean of the fruit. There are, however, considerable variations in cell sizes in different regions of the fruit and it is necessary to examine the gradients in cell size to determine how far the cortical mean cell size represents the value for the fruit as a whole.

(ii) *Cell Size Gradients*.—No satisfactory investigations of the gradients in cell size have been reported, though the variations in different parts of the fruit have been referred to by earlier workers (Smith 1937; Tukey and Young 1942). Five apples from the same tree were taken and weighed; the weights ranged from 120.3 to 216.5 g. These five apples were cut transversely at the equator and the morphological details — cross-sectional area, position of vascular tissue, and size of carpellary cavity — were traced for subsequent measurement. Two transverse sections were then cut from each of four opposite radii in each apple; the first section on each radius extended from the skin to the region of the vascular bundles and the second section extended from the region of the vascular bundles to the carpel wall. From these two sections, it was possible to measure the cells in consecutive fields from the skin to the carpel wall across the fruit; 20 cells from each field were traced and the mean cell volumes were plotted against distance along the radii. Figure 2 shows the cell volume gradients for one of these fruits, which was typical. The cells immediately under the skin are very small but increase in size in the cortex, reaching a maximum size, which then changes little till the region of the vascular tissue is reached, when the cells diminish in size. They increase again in size in the centre of the pith. Cells on either side of the vascular tissue are small and tend to be elongated along a radius of the fruit, especially in the region between the sepal bundle and the dorsal carpellary bundle. Cells of the central pith area are also often considerably elongated along the radius of the fruit. The tendency of the cells of cortex and pith to reach approximately uniform size is apparent. The size of the fruit as a whole will be dependent on the total number of cells in the fruit, and the proportion of that number reaching the maximum size.

Since most of the fruit consists of regions with these cells of maximum size, the mean volume of cells from the cortex can be taken as the volume of cells contributing most to the fruit size. The mean cell volume is given by $3/a^3 \int_0^a v r^2 dr$, where a is the radius of the apple (assumed spherical) and v is the cell volume at distance r from the centre. By inspection of Figure 2, it appears that the cell volume in the mid cortex will be very close to the mean value, and comparison with mean volumes calculated from the formula shows that this is true.

(iii) *Cell Number*.—The "cell number" is calculated by dividing the tissue volume* in the fruit by the mean cell volume estimated from the mid cortex. This is a fair estimate of the number of cells contributing most to the fruit size. This is the same method as used by Smith, except that in his work the cell volume was determined 4 mm. below the skin at the equator.

The "cell number" for the mature fruits of tree 1 in the 1947 and 1948 harvests is plotted against fruit weight in Figure 3. The range of cell numbers is from 17×10^6 in the smallest fruit to 62×10^6 in the largest, i.e. there are

* Tissue volume was determined from the tissue weight divided by the specific gravity of the cells, which was found to average 1.05.

about four times as many cells in the large fruits as in the small fruits. Two straight lines have been fitted to these two sets of data, by the simple linear regression technique, using the usual assumptions and taking fruit weight as the independent variate; the fit is not significantly improved by the inclusion of a quadratic term. The difference between the slopes of the two lines was not significant, but the difference in cell number for fruits of average weight in the two seasons was significant. Over the range of fruit weights investigated, the number of cells per gram was about 160,000 to 170,000. This compares closely with Smith's figure for cell number in Bramley's Seedling. Extrapolation of these lines of best fit is such that the origin is not included in the confidence interval at zero fruit weight. This would support the suggestion that, over the lower range of mature fruit weights, mean cell sizes are lower (cf. Fig. 1).

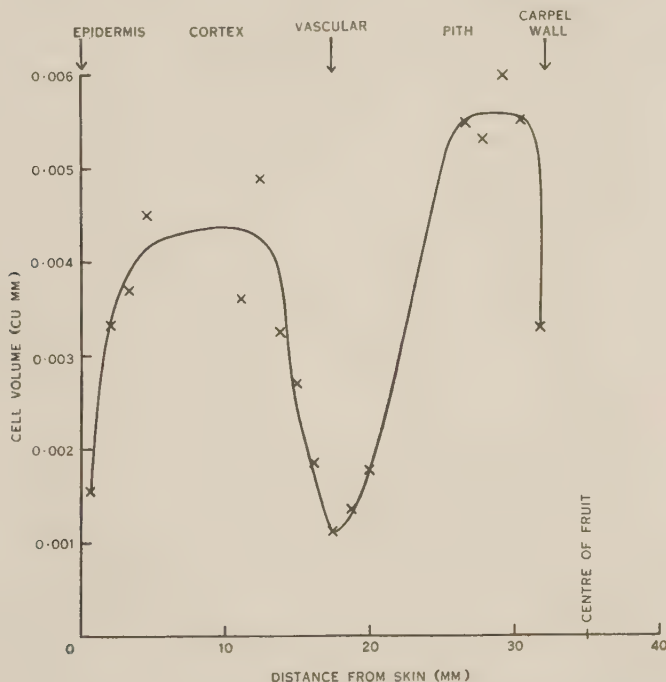


Fig. 2.—Gradient in cell volumes along an equatorial radius in a mature apple.

How far this "cell number" approximates to the true cell number laid down at the time cell division ceases will be discussed in the section on fruit development.

(iv) *Gross Size of Fruit; Cell Size and Air Spaces.*—The overall volume of the fruit is related not only to the number and volume of the cells but also to their packing within the fruit and the volumes occupied by intercellular spaces and carpel cavity. To examine the importance of the air spaces, 25 mature fruits were taken from the 1947 pick over the size range from 160 to 253 g.

and the volumes were determined by displacement. From the weights and volumes the specific gravities of the fruits were obtained; the latter decrease with increasing fruit weight. Using the specific gravity curve against fruit weight as a standard curve, it was possible to calculate the volumes of the fruits used in the main experiment; from this, volume of the tissue, i.e. weight divided by specific gravity of the cells (1.1), was subtracted and the volume of air space in each fruit was obtained. The percentage of air space plotted against fruit weight is shown in Figure 4. The percentage increases rapidly with size of fruit, but in the larger fruits approaches an asymptote at about 27 per cent. of the volume. To eliminate the possibility that this change in proportion of air space was due primarily to an increase in the carpel cavity in large fruits, a number of fruits were peeled and quartered and the carpel region removed. Determination of the specific gravity of the cortical region showed a decrease with increasing weight similar to that obtained with the whole fruit.

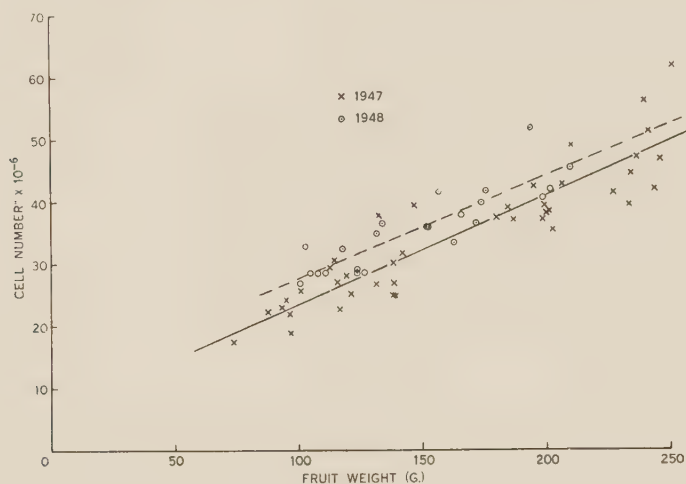


Fig. 3.—Relationship of calculated cell number to fruit weight; apples from the same tree in two seasons; regression equations were $y = 6.0 + 0.17 x$ for 1947 and $y = 11.4 + 0.16 x$ for 1948.

These changes in the relative proportions of cell space and air space indicate a difference in the packing of cells in larger fruit. It is noticeable that the change in percentage of air space is most marked in the smaller size ranges where the fruit size differences are due to cell size differences as well as cell number (cf. Fig. 1). This indicates that the larger the cells, the more loosely they are packed. In the larger size ranges where cell size does not increase with fruit size, the differences in percentage of air space with change in size of fruit are not so marked.

(b) Fruit Growth Experiment

(i) *Cell Number*.—Because of the uncertainty about the validity of the cell number determinations based on the cell sizes of mature fruits, it was thought desirable to estimate cell number on very young fruit to determine the

mean number and the range of numbers to be expected. For this purpose, fruit from tree 2 was used. Cell division was seen clearly in material collected on November 6, 1947. In the dividing cells, the cytoplasm collects in the centre of the cell where the nucleus divides and the two daughter nuclei can be seen clearly in juxtaposition across the new cell wall. The typical appearance of the cells at this stage is shown in Figure 5, which is a camera lucida drawing. By November 10, 1947, cell division appeared to have ceased, i.e. 21 days after full blossom. This agrees with the findings of other workers with other apple varieties. The volumes of the cells in fruit at this early stage were very uniform as the gradients had not yet been established and the cells can be treated as approximately spherical. Table 3 shows the results with these fruits (S.G. taken as 1.1).

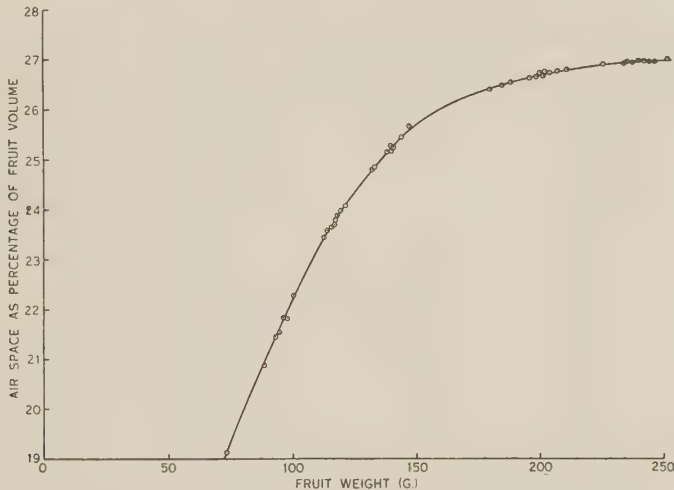


Fig. 4.—Relationship between percentage air space and fruit size.

These results confirm the conclusion that there is a wide range in cell number in these fruits and establish the order of magnitude of the cell numbers in Granny Smith apples. The range in cell number calculated in the above picks was from 21×10^6 to 74×10^6 and the mean cell number was 43×10^6 . Later in the season, a sample of 19 fruits was taken from the same tree on March 17, 1948, i.e. 149 days from full blossom. The cell sizes for the mid-cortical region and the calculated "cell numbers" are shown in Table 4.

The mean calculated cell number at this stage is thus 45.0×10^6 and the range is from 30.6×10^6 to 65.7×10^6 . This is not significantly different from the cell number of the small fruit and indicates that the method of obtaining "cell number" from mean cell volume gives a similar approximation.

(ii) *Gradients of Cell Size Within Fruit.*—Some fruits were examined for gradients in cell size across the equator from skin to carpel cavity, with particular reference to the cortical region. The method was as described for the

fruit size experiment. Figure 6 shows the change in cell diameter in different regions of the cortical tissue, of some of the fruit examined in this way. The gradients seen in the fruit of the fruit size experiment appear to become estab-

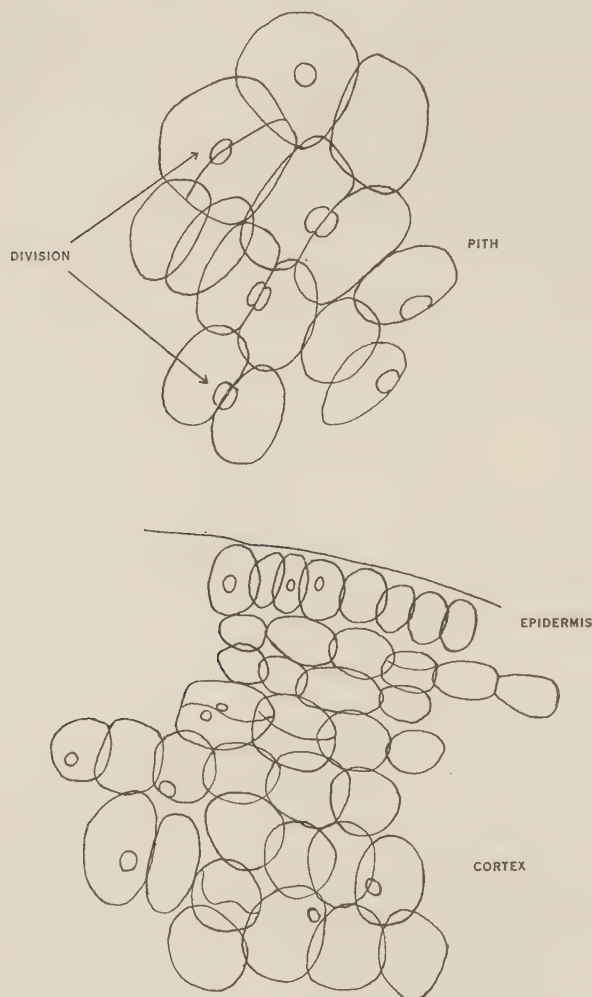


Fig. 5.—Typical appearance of the newly divided cells in young apples.

lished early in the life of the fruit and the cells of the cortex then seem to enlarge in such a way as to preserve the relative differences in size. Thus in fruit 4/10 from pick 9, 15 mm. of cortex contains 60 of the 85 cells (calculated number) with average cell diameter of 0.252 mm., which is significantly different from the mean diameter of the cells in the 4 mm. immediately under the epidermis. By use of these diagrams for gradient of cell diameter, the total number of cells across a given region can be estimated. In this fruit the estimated number is 85, which agrees reasonably with the counted number of 80.

The hypothesis that no cell division occurs after the early period can be tested further by comparing the cortical width, as determined from the area measurements of the whole fruit less that enclosed by the vasculars, with the

TABLE 3
MEAN CELL VOLUMES AND MEAN CELL NUMBERS OF FRUITS IN EARLY SAMPLES

Date of Pick	Days from Full Blossom	Weight of Fruit (g.)	Volume of Tissue (cc.)	Mean Cell* Volume (cu. mm. $\times 10^5$)	Cell Number ($\times 10^{-6}$)
10.xi.47	21	1.40	1.27	2.13	59.5
		1.80	1.64	3.88	42.3
		2.31	2.10	5.10	41.2
		3.40	3.09	6.95	44.5
		4.45	4.05	13.20	30.7
17.xi.47	28	1.20	1.09	2.83	38.5
		1.60	1.45	4.59	31.6
		2.65	2.40	6.08	39.4
		3.15	2.86	4.90	58.5
		3.40	3.09	8.35	37.0
		3.05	2.78	6.01	46.1
		3.70	3.36	9.80	34.2
		3.28	2.98	13.9	21.4
		5.18	4.70	6.31	74.4
		5.75	5.23	9.40	55.7
24.xi.47	35	6.10	5.54	12.40	44.6
		7.35	6.7	15.1	44.3
		7.83	7.1	11.3	62.7
		8.12	7.4	19.6	37.8
		9.60	8.7	19.6	44.3
		8.70	7.9	23.0	34.3
		10.75	9.8	23.0	42.6
		9.07	8.3	19.6	42.3
		12.50	11.4	28.8	39.6
		12.70	11.5	26.8	42.9
		10.20	9.3	19.6	47.5
		11.42	10.4	40.8	25.4
		14.10	12.8	31.1	41.1
		12.45	11.3	28.0	40.4
1.xii.47	42	13.55	12.3	25.6	47.0
		14.15	12.7	32.6	38.7
		14.95	13.6	51.2	26.6
		17.35	15.7	55.6	28.2
		19.30	17.5	40.2	43.5
		15.45	14.0	31.6	44.3
		16.50	15.0	32.0	46.9
		20.95	19.1	39.8	48.0

* Except for the first pick, where 200 cells were measured, 100 cells were measured in each.

calculated cortical width based on the total assumed number of cells (78) and the mean major axis of the cells measured. The value of 78 cells in the cortex was the mean counted number in pick 1 when the cells were still countable. These results are given in Table 5.

The agreement between the observed and calculated trend in cortical thickness is in reasonable agreement with the hypothesis that cell enlargement alone is responsible for the increase in width of the cortex. Fruit of pick 9, which will be discussed later, departs anomalously. It should be pointed out

TABLE 4
MEAN CELL VOLUMES AND MEAN CELL NUMBERS OF FRUITS TAKEN LATE IN THE
SEASON FROM THE SAME TREE AS THOSE SHOWN IN TABLE 3; DATE OF PICK
MARCH 17, 1948, 149 DAYS FROM FULL BLOSSOM

Weight of Fruit (g.)	Volume of Tissue (cc.)	Mean Cell Volume (cu. mm. $\times 10^4$)	Cell Number ($\times 10^{-6}$)
142.02	129	36	35.9
161.51	147	48	30.6
161.41	146	38	38.5
163.10	148	39	38.0
167.46	152	39	39.0
172.31	156	37	42.2
174.15	158	39	40.5
177.50	162	36	45.0
180.20	164	47	34.9
185.14	168	34	49.5
188.20	171	40	42.7
189.50	173	36	48.0
200.10	182	39	46.7
201.07	183	45	40.7
202.85	185	32	57.9
205.72	187	43	43.5
222.10	202	34	59.5
239.16	217	33	65.7
249.91	297	40	56.5

that a small variation in the number of cells across the cortex, however, represents considerable increase in cell number. The average radius for fruit of pick 8 is 3.91 cm. Compare a spherical fruit (of average radius) with 80 cells

TABLE 5
RELATION BETWEEN WIDTH OF CORTEX AS MEASURED AND WIDTH OF CORTEX AS
CALCULATED FROM MEAN MAJOR AXIS OF CELLS

Pick	Av. Weight (g.)	Av. Width of Cortex (cm.)	Mean Major Axis of Cells (mm.)	Calculated Width (cm.)
1	19.85	0.70	0.098	0.76
2	42.20	0.87	0.132	1.03
3	96.70	1.40	0.184	1.44
4	119.20	1.39	0.182	1.42
5	149.47	1.74	0.206	1.61
6	180.38	1.79	0.226	1.76
7	201.45	1.84	0.220	1.72
8	208.82	1.88	0.248	1.93
9	214.0	1.86	0.292	2.28

across the cortex with another spherical fruit having 90 cells across the cortex. The average radius for each cell for that pick is 0.011 cm. Then the radius of the larger fruit would be 4.13 cm. and the volumes of the two spheres would be 250 and 295 cc., the additional rows of cells contributing 45 cc. Such an addition of 10 rows of cells, i.e. an increase of $12\frac{1}{2}$ per cent., would increase the total cell number of the fruit from 30×10^6 to 35.4×10^6 , or an increase of 18 per cent.

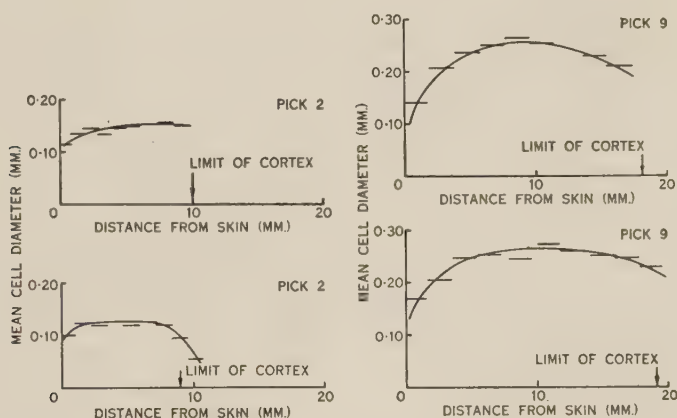


Fig. 6.—Gradient in cell size in the cortex of a young fruit (72 days from full blossom) compared with that of a mature fruit (205 days from full blossom).

So far considerations have been based on the increase in width at the equator of the fruit. Some measurements of gradients in cell size in other directions in the fruit were made in the development experiment. Observations showed that the gradients in the cortex, along radii other than those at the equator, did not differ significantly from those at the equator. It seems, therefore, that the measurements taken at the equator are likely to be satisfactory for obtaining mean cell volume.

(iii) *Gross Development and Cell Size*.—The development of the fruit, as measured by weight and plotted against number of days from full blossom, is given in Figure 7. This gives the usual form of growth curve. No direct measurements of volume were made on most of these picks. Simultaneously, the areas of the fruits across the equators were measured and these areas were used to determine a mean radius. The volumes of spheres of these radii were calculated and these are also plotted for comparative purposes in Figure 7 as the fruit "volume." The volumes of the fruits used in picks 9 and 10 were determined and are also shown on the graph; the calculated spherical volume underestimates the actual volume.

Within the fruit, the comparative areas of the different regions of tissue at the equator, as seen in transverse section, are given in Figure 8. Areas increase up to approximately 190 days from full blossom (mean weight 207 g.) and this increase is shown in both cortex and pith. The area of the carpel

cavities altered little with increasing fruit size, contributing little to the total area and therefore little to the total volume. The relative proportions of cortex and pith did not change very much; the cortex increased from 64 per cent. of the area in 20 g. fruit to 73 per cent. of the area in 214 g. fruit; meantime the pith decreased from 27.6 per cent. to 24.3 per cent. The relative proportions of the different tissues were constant after 135 days from full blossom (mean weight 150 g.).

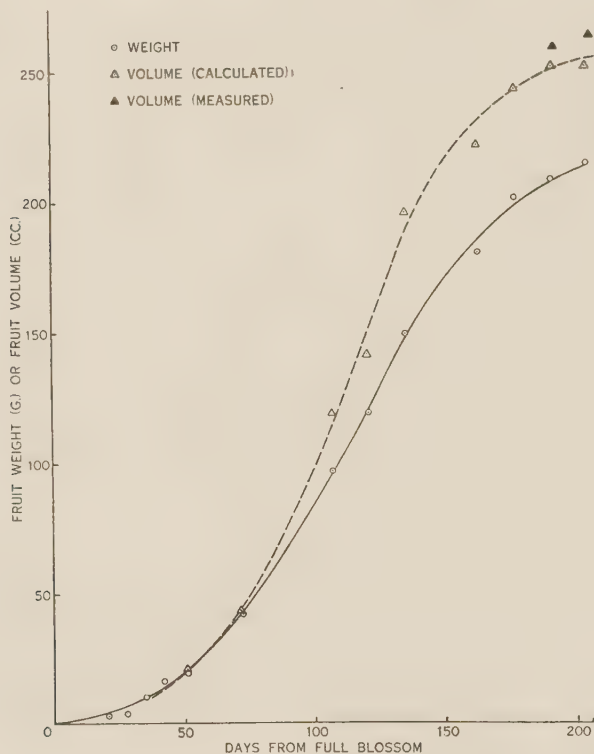


Fig. 7.—Increase in mean weights of fruits with time; calculated fruit "volumes" are included for comparison with the measured volumes of two samples.

Since all the evidence supports the view that, after the first few weeks, fruit growth is a matter of cell enlargement, provided that the specific gravity of the cells does not change during development, the applicability of the cell size determinations to the three-dimensional development of the fruit can be obtained from calculation of cell volumes and comparison with fruit weight. This comparison is better than that with fruit volume, which may be complicated by changes in the intercellular spaces as well as changes in cell volume. The specific gravity of the cells and their contents, which was shown to be about 1.05, does not alter markedly during the developing period. Thus volumes of cells can be compared with the weight of the fruit without introducing an error of any great magnitude. When the mean cell volumes of each

pick are plotted against mean fruit weights, a linear relationship between calculated cell volume and fruit weight is obtained over most of the range of sizes, though there is a suggestion that pick 9 departs anomalously (Fig. 9). The results are given in Table 6.

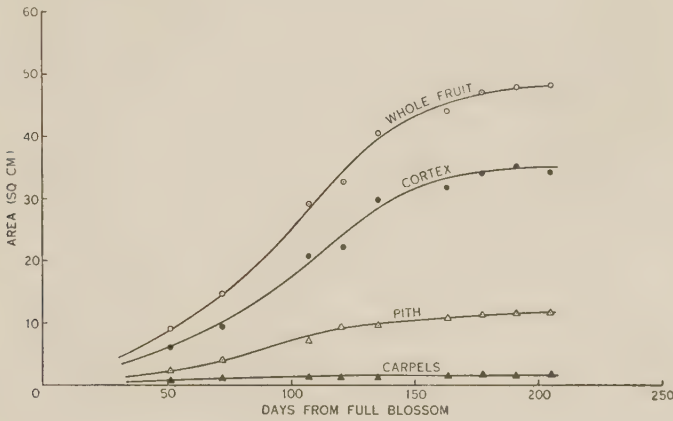


Fig. 8.—Changes in area of different tissues with time.

From the preceding discussion, it would be expected that, if the sample of 10 fruits from each tree were large enough to represent truly the cell sizes and cell numbers of the fruit on the tree at the time of picking, the relationship would be perfectly linear. The fruits of pick 9 give an indication of what

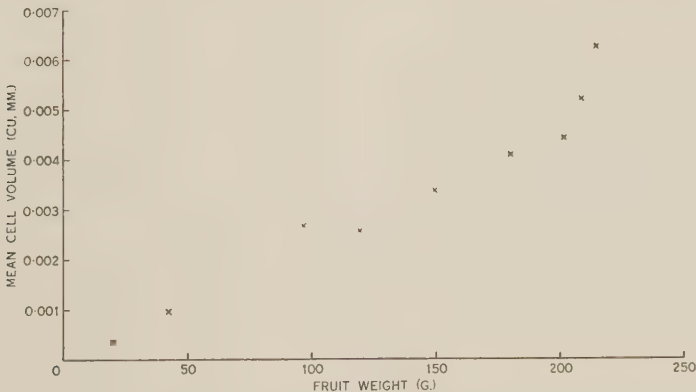


Fig. 9.—Mean volumes of mid-cortical cells of successive samples plotted against fruit weight.

is probably the principal cause of this departure. It can be seen from Figure 10 that this sample contains a wider range of size classes than either of the two preceding samples. Despite this, the cell volumes of the smaller fruits in this pick are as great as those of the larger fruits, therein resembling the cell size range in the fruit size experiment. If the cell size is used to calculate cell

number for pick 9, the same wide range in number that has been noted earlier is found. Thus the cell number ranges from 21.3×10^6 to 46.1×10^6 with a mean at 32.6, which is significantly different from the mean of pick 8. This seems to indicate that the sampling of 10 fruits per tree is not satisfactory to

TABLE 6
RELATIONS BETWEEN MEAN CELL VOLUME AND MEAN CELL NUMBER IN FRUITS
OF SUCCESSIVE PICKS

Pick	Days from Full Blossom	Av. Weight (g.)	Tissue Volume (cc.)	Mean Cell Volume (cu. mm.)	Mean Cell Number ($\times 10^{-6}$)
1	51	19.9	18.9	0.00037	51.0
2	72	42.2	40.2	0.00096	41.8
3	107	96.7	92.0	0.00267	34.5
4	121	119.2	113.8	0.00255	44.6
5	135	149.5	142.2	0.00337	42.3
6	163	180.4	172.0	0.00409	42.1
7	177	201.5	191.8	0.00442	43.4
8	191	208.8	199.0	0.00521	38.2
9	205	214.6	204.5	0.00628	32.6

give a true picture of the relation of cell enlargement by volume to the total weight of the fruit, because the relationship may be obscured by the wide range in cell number.

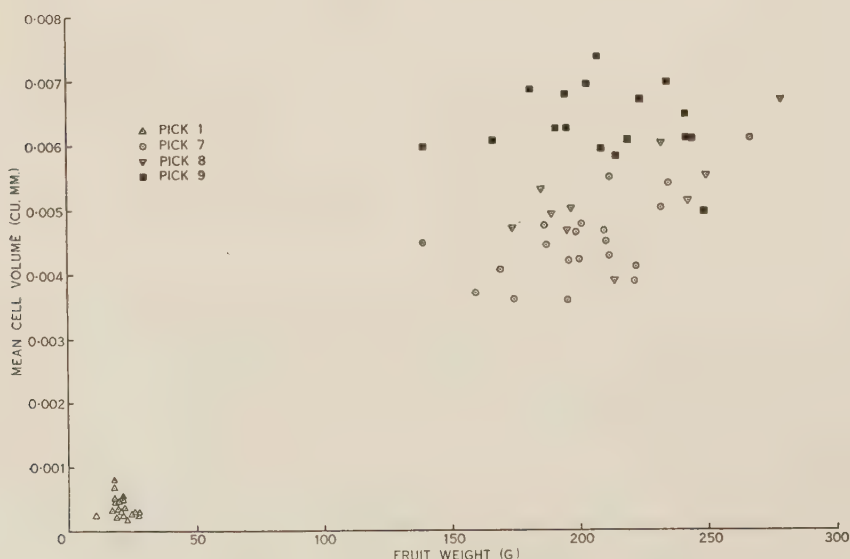


Fig. 10.—Mean values of mid-cortical cells of individual fruits in different samples plotted against fruit weight.

The results show that, as long as the fruit stays on the tree, cell enlargement continues, and at the end of this experiment the cell size was still increasing. The change in mean cell size with time from full blossom is plotted in Figure 11.

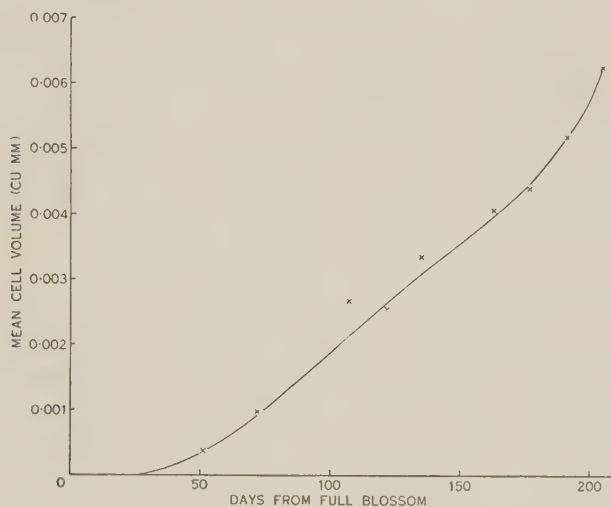


Fig. 11.—Mean volumes of mid-cortical cells of successive samples plotted against time from full blossom.

IV. ACKNOWLEDGMENTS

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V. REFERENCES

- MACARTHUR, M., and WETMORE, R. H. (1939).—*J. Pomol.* 17: 218.
 MACARTHUR, M., and WETMORE, R. H. (1941).—*Canad. J. Bot.* 19: 371.
 MACDANIELS, L. H. (1940).—*Cornell Agric. Exp. Sta. Mem.* No. 230.
 SMITH, W. H. (1937).—Report of Food Investigation Board for the year 1937, p. 127.
 SMITH, W. H. (1940).—*J. Pomol.* 18: 249.
 SMITH, W. H. (1950).—*Ann. Bot. N.S.* 14: 23.
 TETLEY, U. (1930).—*J. Pomol.* 8: 153.
 TETLEY, U. (1931).—*J. Pomol.* 9: 278.
 TUKEY, H. B., and YOUNG, J. O. (1942).—*Bot. Gaz.* 104: 3.

THE PHYSIOLOGY OF GROWTH IN APPLE FRUITS

II. RESPIRATORY AND OTHER METABOLIC ACTIVITIES AS FUNCTIONS OF CELL NUMBER AND CELL SIZE IN FRUIT DEVELOPMENT

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Summary

The relationship of respiratory activity and quantities of the chemical constituents to fruit size, cell size, and cell number in apple fruits of the variety Granny Smith were examined.

In an experiment on mature fruits, size of fruit was primarily due to cell number, but larger cells contained more protein nitrogen than smaller cells. Soluble nitrogen and protein nitrogen were strongly correlated. Correlations between respiration rate per cell and cell volume, and between respiration rate per cell and protein nitrogen per cell were low.

In a fruit growth experiment, changes in carbohydrate fractions, organic acids, protein and total nitrogen, cell wall material, and respiration rate were compared on a unit cell basis. Sugars, particularly sucrose, increased markedly during growth, starch increased at first and decreased subsequently, and cell wall substances increased in proportion to increase in cell surface. Soluble and protein nitrogen increased together, more in relation to increase in cell surface than to increase in cell volume; this is interpreted as an increase in cytoplasm of approximately constant thickness during the enlargement of the vacuole.

Respiration rate which, after a slight decrease, increased in proportion to increase in protein nitrogen, finally increased more rapidly than protein nitrogen at the time that starch was disappearing and unknown organic acids were increasing rapidly compared with malic and citric acids; the bearing of these observations on a new hypothesis of the cause of the climacteric rise in respiration is discussed.

I. INTRODUCTION

The chemical changes in carbohydrates, pectins, acids, and nitrogen fractions associated with the development of apple fruits have been investigated by a number of workers (e.g. Archbold 1932; Hulme 1936; Krotkov and Helson 1946). These investigations have defined the relation of the changes to respiration rates. Little has been done to relate these changes to the cellular development of the fruit; the relationship has been discussed in principle by Kidd (1934), and the relationships between cell development, protein content, and respiration rate have been examined by Hulme and Smith (1938). The physiological approach is possible only when the relationships of metabolism and cell development have been defined; in this paper we shall attempt to define these problems, and to take account of recent knowledge of relevant biochemical

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processes (e.g. nitrogen metabolism, the role of organic acids, and phosphorylation) that were less well understood when the earlier surveys were carried out.

Much has been written on the factors regulating protein level in plant tissues, particular emphasis being laid on the possible correlation between protein content and respiration rate. Apple tissue has some advantages for a study of such a relationship since cell division ceases at an early stage, and protein synthesis continues through the period of enlargement, accompanying the entry of carbohydrate and soluble nitrogen and the increase in organic acids. At the same time, there is a spectacular increase and a subsequent decrease in the starch content. A marked rise in respiration, which occurs at about this time, is characteristic of certain varieties of apples, and requires further investigation before it can be satisfactorily explained. With so many changes occurring concurrently, the developing apple is very suitable material for contributing to our knowledge of the interrelationships of such processes in plant tissue.

In this paper, data obtained by analyses on material from the same source as that used for the anatomical study discussed in the preceding paper (Bain and Robertson 1951) are presented. The experimental data therefore have been used for two main purposes—to relate gross fruit size, cell size, and cell number to the soluble nitrogen and protein levels and to the respiration rates of fruits at maturity, and to relate the increase in size of the cells of the fruit to the changes in carbohydrates, nitrogen fractions, organic acids, and respiration rates.

II. MATERIAL AND METHODS

Two experiments were carried out on the same Granny Smith apples as those used for the anatomical examinations.

In the experiment on size in mature fruits, the fruits taken from tree 1 at Orange, N.S.W., in 1947 and 1948, were used for individual respiration rate measurements (over several days) before the sections for the cell size determinations were cut. Each fruit was enclosed in a chamber and the respired CO_2 estimated by the Pettenkofer method, using $0.1\text{N Ba}(\text{OH})_2$. Subsequently, after the removal of the anatomical specimens, the flesh of the 1947 fruits (i.e. with skin and carpels removed) was used for the determination of total and protein nitrogen in each fruit by a method previously described by Turner (1949). In 1947, the respiration rate was determined at 25°C .; in 1948, the respiration rate was determined at 21°C ., but the rates were corrected to 25°C ., assuming a temperature coefficient (Q_{10}) of 2, and as they were similar to the 1947 results, are not given here.

In the fruit growth experiment, the samples used for analysis were replicates, each of the same number of fruits as those used for the anatomical investigation. Table 1 describes these samples.

The respiration of the bulk sample was determined at 20.0°C . Subsequently, the bulked flesh of the fruits of each sample was analysed for total sugars, reducing sugars, non-reducing sugars ("sucrose"), starch, alcohol-insoluble residue, total organic acids, malic acid, citric acid, total nitrogen,

and protein nitrogen. Changes in fresh and dry weight were determined. The methods of tissue preparation and analysis were similar to those described by Turner (1949).

The cell size and cell number data for the replicate samples in the previous paper were used as the basis for the samples in this experiment. Analytical data are expressed as g. per fruit or as g. per cell, unless otherwise stated.

III. RESULTS

(a) *Size in Mature Fruits*

The respiration rates, total protein nitrogen, and soluble nitrogen (the difference between total and protein nitrogen), together with the estimated number of cells per fruit (Bain and Robertson 1951), are given in Table 2.

Comparison of these figures shows that, as fruit weight increases, protein nitrogen, cell number, and respiration also increase. Partial correlation coefficients for the different factors have been determined and are given in Table 3.

Protein nitrogen, soluble nitrogen, respiration rate, and cell number are all positively correlated with fruit weight. Soluble nitrogen and protein nitrogen are positively correlated. Respiration rate is not correlated with protein nitrogen, soluble nitrogen, or cell number, and protein nitrogen is not correlated with cell number. These correlations suggest that the protein content of these apples could have been governed primarily by the concentration of soluble nitrogenous constituents.

TABLE 1
DATE OF PICKING, DAYS FROM FULL BLOSSOM, AND MEAN WEIGHT OF SUCCESSIVE
SAMPLES FROM TWO TREES

Pick	No. of Fruits		Date	Days from Full Blossom	Mean Weight (g.)
	Tree 3	Tree 4			
1	20	—	10.xii.47	51	21.2
2	20	—	31.xii.47	72	42.4
3	10	10	4.ii.48	107	99.1
4	10	10	18.ii.48	121	120.7
5	10	10	3.iii.48	135	155.1
6	10	10	31.iii.48	163	187.3
7	10	10	14.iv.48	177	207.8
8	10	10	28.iv.48	191	209.6
9	9	9	12.v.48	205	228.3

Since the mean cell numbers and mean cell volumes (particularly for the flesh region) are known, it is possible to examine the relationship between protein nitrogen per cell, respiration per cell, and cell volume. These figures are given in Table 4. When these relations are examined, the correlations shown in Table 5 are obtained.

The low correlation between the respiration per cell and the protein nitrogen per cell, while positive and significant at the 5 per cent. level, does not suggest that, in these fruits, these two factors are closely related. This does

TABLE 2

RELATIONSHIPS OF RESPIRATION RATE, NITROGEN FRACTIONS, CELL VOLUME, AND CELL NUMBER IN A POPULATION OF MATURE FRUITS OF DIFFERENT SIZES FROM ONE TREE

Fruit Weight (g.)	Respiration per Fruit (mg. CO ₂ /hr. at 25°C.)	Nitrogen per Fruit (g.)			Mean Cell Volumes (cu. mm.)	No. Cells per Fruit (× 10 ⁻⁶)
		Total	Protein	Soluble		
73.8	—	—	—	—	0.0039	17.2
87.7	2.19	0.0146	0.0127	0.0019	0.0036	22.2
93.4	2.62	0.0192	0.0141	0.0051	0.0037	22.9
94.5	—	0.0212	0.0162	0.0050	0.0036	23.9
96.2	2.51	0.0195	0.0137	0.0058	0.0040	21.9
96.8	2.51	0.0216	0.0143	0.0073	0.0047	18.7
101.0	2.07	0.0284	0.0187	0.0097	0.0036	25.6
112.7	2.51	—	—	—	0.0035	29.3
114.1	3.06	0.0212	0.0164	0.0048	0.0034	30.5
115.5	2.82	0.0380	0.0211	0.0169	—	—
115.7	2.65	0.0289	0.0200	0.0089	0.0039	27.0
116.9	1.96	0.0288	0.0195	0.0093	0.0047	22.6
118.1	2.80	0.0233	0.0163	0.0070	—	—
119.8	2.28	0.0193	0.0158	0.0035	0.0039	28.0
121.4	2.37	0.0245	0.0174	0.0071	0.0044	25.1
131.9	2.96	0.0228	0.0193	0.0035	0.0045	26.6
132.4	3.26	0.0259	0.0182	0.0077	0.0032	37.6
138.4	3.06	0.0345	0.0217	0.0126	0.0051	24.7
138.4	2.99	—	—	—	0.0042	30.0
138.7	2.69	—	—	—	0.0047	26.8
139.2	2.42	0.0381	0.0222	0.0159	0.0051	24.8
142.3	3.18	0.0353	0.0229	0.0124	0.0041	31.6
146.9	2.97	0.0264	0.0215	0.0049	0.0034	39.3
180.1	3.92	0.0742	0.0334	0.0408	0.0044	37.4
184.8	3.83	0.0445	0.0327	0.0118	0.0043	39.0
187.3	4.08	0.0342	0.0277	0.0065	0.0046	37.0
195.1	4.22	0.0385	0.0289	0.0096	0.0042	42.3
198.7	4.38	0.0455	0.0314	0.0141	0.0049	37.0
199.6	3.56	0.0428	0.0266	0.0162	0.0046	39.4
200.4	3.34	0.0421	0.0311	0.0110	0.0048	38.0
200.9	3.65	0.0635	0.0344	0.0291	0.0048	38.2
203.1	3.90	0.0403	0.0303	0.0100	0.0052	35.4
206.7	4.03	0.0357	0.0282	0.0075	0.0044	42.7
210.1	3.94	0.0417	0.0323	0.0094	0.0039	49.0
227.2	4.91	0.0583	0.0372	0.0211	0.0050	41.3
233.6	4.84	0.0373	0.0315	0.0058	0.0054	39.3
234.5	4.45	—	—	—	0.0048	44.4
236.7	4.45	0.0484	0.0350	0.0134	0.0046	47.0
239.4	4.52	0.0555	0.0399	0.0156	0.0039	56.0
241.5	4.06	0.0497	0.0364	0.0133	0.0043	51.1
243.7	4.75	0.0537	0.0382	0.0155	0.0053	41.9
245.9	4.61	0.0731	0.0421	0.0310	0.0048	46.6
251.1	4.78	0.0430	0.0352	0.0077	0.0037	61.7

In working out partial correlation coefficients, information in incomplete rows has not been included.

not mean that these fruits differ from those used by Hulme and Smith (1938); they also found a departure in mature fruits from the high correlation between protein nitrogen per cell and respiration per cell shown in younger fruits. This departure they attributed to the rise in respiration associated with the climacteric. This will be discussed further in reference to the fruit development experiment.

From this experiment it is concluded that large fruits have more protein nitrogen than small, and that this is due partly to the larger numbers of cells, but the protein nitrogen per cell is strongly correlated with the cell volume. The amount of protein nitrogen may be regarded as a measure of the cytoplasmic content but is not strongly correlated with respiration rate in the later stages of maturity.

(b) Fruit Growth Experiment

(i) Fresh Weight and Dry Weight

The mean fresh weights per fruit in the different samples were not significantly different from those obtained with the samples used for the cell size determinations. The fresh weight and dry weight curves were the usual type of sigmoid curve obtained in growth studies (see Gustafson 1926); similar curves for apples have been obtained by Askew (1935) and Hulme (1936). Archbold (1932), however, obtained a linear increase in weight of Bramley's Seedling and Worcester Pearmain apples over most of the growing period.

TABLE 3
PARTIAL CORRELATIONS FOR THE DIFFERENT FACTORS: 1, FRUIT WEIGHT; 2, RESPIRATION RATE; 3, PROTEIN NITROGEN; 4, SOLUBLE NITROGEN; 5, CELL NUMBER

Factors Examined	Partial Correlation	Probability
$r_{12 \cdot 345}$	0.4220	0.02 > P > 0.01
$r_{13 \cdot 245}$	0.7783	0.001 > P
$r_{14 \cdot 235}$	0.4784	0.01 > P > 0.001
$r_{15 \cdot 234}$	0.3188	0.1 > P > 0.05
$r_{23 \cdot 145}$	0.0218	P > 0.9
$r_{24 \cdot 135}$	0.0044	P > 0.9
$r_{25 \cdot 134}$	0.0659	P > 0.7
$r_{34 \cdot 125}$	0.7535	0.001 > P
$r_{35 \cdot 124}$	0.0664	0.8 > P > 0.7
$r_{45 \cdot 123}$	0.2003	0.3 > P > 0.2

In view of the observations on cell size and cell number in the samples of 20 fruits used for the anatomical work, it is necessary to examine the significance of a "growth" curve obtained from the fresh and dry weights. The growth curve would be a valid representation of what happened in time only if the mean number of cells in the different samples were constant and this has been shown to be untrue (Bain and Robertson 1951). Using the mean cell numbers obtained in that work, the growth curve expressed as dry weight per cell (Fig. 1), becomes of the same form as the cell volume curve from

which the cell numbers were calculated. Dry weight per cell increases relatively more rapidly than cell volume, especially near the end of the growing

TABLE 4

RELATIONSHIPS OF CELL VOLUME, CELL NUMBER, RESPIRATION RATE PER CELL, AND PROTEIN NITROGEN PER CELL IN MATURE FRUITS FROM ONE TREE

Fruit Weight (g.)	Mean Cell Volume (cu. mm.)	Mean Cell Number ($\times 10^{-6}$)	Protein Nitrogen per Cell (mg. $\times 10^{-7}$ /cell)	Respiration per Cell (mg. $\times 10^{-7}$ CO ₂ /cell/hr.)
73.8	0.0039	17.2	—	—
87.7	0.0036	22.2	5.72	0.99
93.4	0.0037	22.9	6.16	1.14
94.5	0.0036	23.9	6.78	—
96.2	0.0040	21.9	6.26	1.15
96.8	0.0047	18.7	7.65	1.34
101.0	0.0036	25.5	7.33	0.81
114.1	0.0035	30.5	5.37	1.00
115.7	0.0039	27.0	7.40	0.98
116.9	0.0047	22.6	8.63	0.87
119.8	0.0039	28.0	5.65	0.82
121.4	0.0044	25.1	6.93	0.94
131.9	0.0045	26.6	7.25	1.11
132.4	0.0032	37.6	4.85	0.96
138.4	0.0051	24.7	8.86	1.24
138.4	0.0042	30.0	—	1.00
138.7	0.0047	26.8	—	1.00
139.2	0.0051	24.8	8.95	0.98
142.3	0.0041	31.6	7.25	1.01
146.9	0.0034	39.3	5.47	0.76
180.1	0.0044	37.4	8.93	1.05
184.8	0.0043	39.0	8.40	0.98
187.3	0.0046	37.0	7.49	1.10
195.1	0.0042	42.3	6.89	1.00
198.7	0.0049	37.0	8.48	1.19
199.6	0.0046	39.4	6.76	0.91
200.4	0.0048	38.0	8.17	0.88
200.9	0.0048	38.2	9.00	0.96
203.1	0.0052	35.4	8.56	1.10
206.7	0.0044	42.7	6.60	0.94
210.1	0.0039	49.0	6.60	0.80
227.2	0.0050	41.3	9.00	1.19
233.6	0.0054	39.3	8.01	1.23
234.5	0.0048	44.4	—	1.00
236.7	0.0046	47.0	7.45	0.95
239.4	0.0039	56.0	7.13	0.81
241.5	0.0043	51.1	7.12	0.80
243.7	0.0053	41.9	9.10	1.13
245.9	0.0048	46.6	9.03	0.99
251.1	0.0037	61.7	5.79	0.78

season, which emphasizes how unsatisfactory increase in dry weight would be as a measure of fruit growth. The dry weight as a percentage of the fresh

weight of the fruit increased appreciably, from 10.6 to 15.2 per cent., during the period of sampling. The significance of this increase in dry weight will be discussed subsequently.

TABLE 5

CORRELATION BETWEEN PROTEIN NITROGEN PER CELL, RESPIRATION RATE PER CELL, AND CELL VOLUME

Correlated Factors	Coefficient	Probability
Protein nitrogen per cell : cell volume	0.84	$0.01 > P > 0.001$
Respiration per cell : cell volume	0.53	$0.01 > P > 0.001$
Respiration per cell : protein nitrogen per cell	0.39	$0.05 > P > 0.01$

(ii) Carbohydrates

Sugars.—Most of the changes in dry weight in apple tissue are due to the increase in the carbohydrates during development. The sugar content per cell increases markedly during growth (Fig. 1). In early samples, sugars, consisting almost entirely of reducing sugars, are at a low level, accounting for 24.1 per cent. of the dry weight, but total sugars increase markedly in the

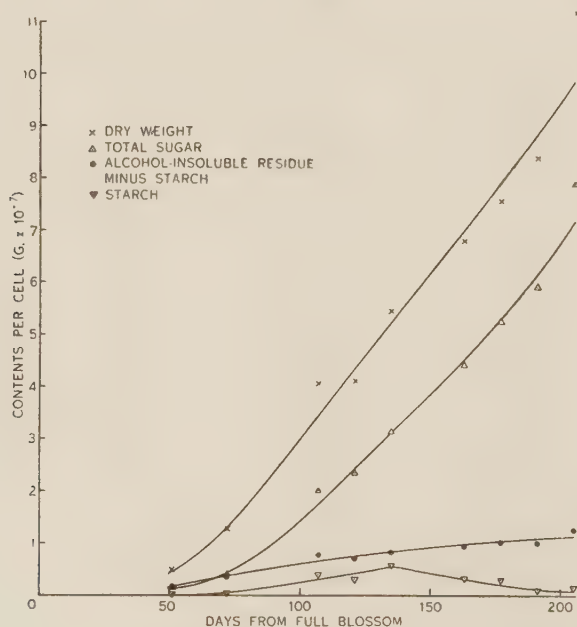


Fig. 1.—Changes in dry weight, total sugar, wall substance, and starch per cell, with time.

later samples till, in the final sample, they amount to 71.0 per cent. of the dry weight and 10.8 per cent. of the fresh weight. Sucrose, which is present in negligible amounts in the early samples, increases in the later samples and accounts for 26.2 per cent. of the dry weight in the final sample.

Thus the cell possesses the power of importing a considerable quantity of carbohydrate up to the end of the season and of retaining the bulk of this imported material in soluble form. These observations are in general agreement with those obtained by others with other varieties.

Starch.—This increases little between the first two samples, but subsequent samples show a marked increase till, after the fifth sample, 135 days from full blossom, the starch content decreases (Fig. 1). Similar changes in starch content have been observed by a number of workers, particularly by Widdowson (1932) with Bramley's Seedling and Worcester Pearmain apples. The significance of this rise and fall in starch content will be discussed subsequently.

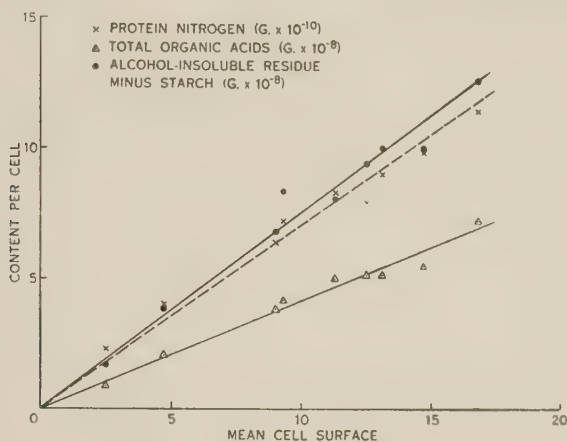


Fig. 2.—Relationship between the protein nitrogen, total organic acids, and cell wall substances per cell and cell surface.

Alcohol-insoluble residue minus starch per cell.—This increases throughout the experiment (Fig. 1), though less rapidly in later samples than any other of the carbohydrate constituents. Since the major components of this fraction are probably cell wall constituents, pectic compounds, cellulose, hemicelluloses, etc. (Widdowson 1932), it is interesting to examine the change in these constituents with increase in the size of the cells of the fruit. In Figure 2, the alcohol-insoluble residue minus starch is plotted against the mean surface area of the cells, calculated on the basis of the mean values for the major and minor cell axes for each pick, and using the formula

$$A = 2\pi b^2 + 2\pi \frac{ab}{e} \sin^{-1} e,$$

where A is the surface area of the cell, a and b are the major and minor semi-axes respectively, and e ($= \sqrt{1 - b^2/a^2}$) is the eccentricity. It can be seen that cell wall material increases linearly with cell surface throughout the growth of the cells, indicating that the cell wall remains approximately constant in thickness and increases in area.

(iii) *Nitrogen Compounds*

Total nitrogen.—The changes in total nitrogen per fruit in the different samples would give a curve similar to that based on fresh weight measurements. These data are in agreement with the observations of other workers using a number of varieties (Archbold 1932; Askew 1935; and Hulme 1936).

When the results with Granny Smith apples are expressed as the total nitrogen per cell, a different picture is obtained (Fig. 3). The rate of uptake per cell changes little during growth so that there is an increase in the amount of nitrogen per cell, even in the last two samples (191 and 205 days from full blossom), which, on the whole fruit basis, appeared to show a slight decrease. Thus the uptake of nitrogen per cell continues throughout the developing period of the cells of the fruits.

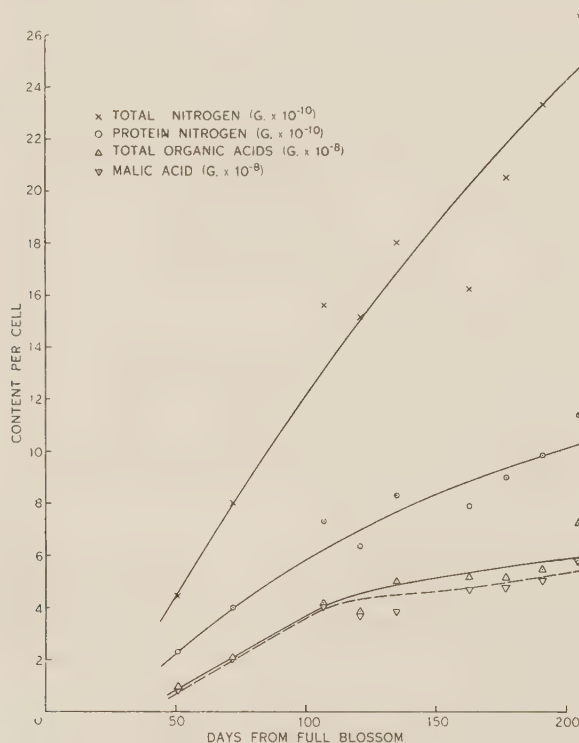


Fig. 3.—Changes in total and protein nitrogen and total and malic acids per cell, with time.

Protein nitrogen.—The protein nitrogen content parallels closely the total nitrogen content per fruit, rising from 0.0112 g. per fruit in the first sample to 0.0333 g. per fruit in the fifth sample (135 days from full blossom), with little subsequent increase and no significant change after the seventh sample (177 days). These agree with the only comparable data published (Hulme 1936).

The protein nitrogen per cell also follows the total nitrogen per cell, continuing to increase throughout the season (Fig. 3). If it is assumed that the

protein nitrogen content is an approximate measure of the cytoplasmic content of the cells, it seems reasonable to expect a relationship to the cell surface on the assumption that the cytoplasmic lining of the cell increases with the cell surface during growth, but remains approximately constant in thickness. In Figure 2 the protein nitrogen per cell is plotted against the mean cell surface and a linear relationship is obtained. This would support the suggestion that the cytoplasm increases with constant thickness as cell volume increases.

Soluble nitrogen.—When the figure for protein nitrogen is subtracted from that for total nitrogen, the difference can be taken as the soluble nitrogen which, with the protein nitrogen, is shown in Table 6. If a linear regression is

TABLE 6
RELATIONSHIP BETWEEN PROTEIN NITROGEN AND SOLUBLE NITROGEN IN
SUCCESSIVE PICKS

Pick	Protein Nitrogen per Fruit (g.)	Soluble Nitrogen per Fruit (g.)
1	0.0112	0.0097
2	0.0156	0.0156
3	0.0238	0.0277
4	0.0257	0.0354
5	0.0333	0.0390
6	0.0321	0.0326
7	0.0375	0.0479
8	0.0361	0.0491
9	0.0354	0.0477

assumed, the two quantities are correlated with a coefficient of 0.968, significant at the 0.1 per cent. level. Calculations on the data presented by Hulme (1936) also reveal highly significant correlations between protein and soluble nitrogen content per fruit. As was discussed in the first part of the paper, this relationship is also seen in individual fruits taken at maturity.

It is important to note that Hulme showed that 90 per cent. of the soluble nitrogen (alcohol extraction) of Bramley's Seedling apples is accounted for by amino acids and asparagine. Amino acids and asparagine have been shown qualitatively to form important constituents of the soluble nitrogen of Granny Smith apples (McKee, unpublished data).

(iv) Organic Acids

Total organic and malic acids.—The changes in total organic acids and in malic acid per cell are shown in Figure 3. Malic acid, accounting for nearly all the total organic acids in the earlier picks, closely follows the rise in total acids until pick 5 (135 days). After this, however, the trend in malic acid shows a significant deviation from that of the total organic acids and later samples have larger amounts of other organic acids.

The total organic acids per cell increase rapidly at first and less rapidly later, but continue to increase throughout the samples. The curve resembles the curve for protein nitrogen per cell and this is most clearly seen when the

total organic acids per cell are plotted against cell surface (Fig. 2). The fact that the organic acids per cell are so highly correlated with the protein nitrogen per cell raises the interesting possibility of the interdependence of these two constituents in a steady state relationship. Although there is available as yet little knowledge of the metabolic processes underlying the growth of the apple, there is some evidence (Turner 1949) that a mechanism akin to the tricarboxylic acid cycle of Krebs (1943) may function in carbohydrate metabolism in the mature Granny Smith apple. If such a cycle operates in the developing fruit, the organic acids may be expected to be related in amount to the level of the protein and soluble nitrogen, since they form the connecting link with the carbohydrate metabolism. This relationship will be a fruitful field for further investigation.

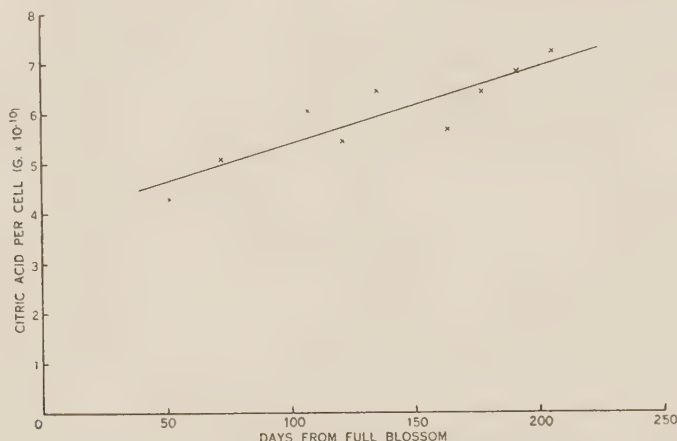


Fig. 4.—Changes in citric acid per cell with time.

Citric acid.—Citric acid (Fig. 4) behaves rather differently; there is relatively little change in the amount present per cell (4.28×10^{-10} g.) from the time of the initial sample until the third sample (6.06×10^{-10} g.) 107 days later, during which time both total and malic acids increase more than threefold. Citric acid then continues to increase, coinciding with the appearance of appreciable amounts of organic acids other than citric and malic. Up to about 121 days from full blossom, malic and citric acids, within the limits of experimental error, account for practically all the total organic acids present. After this, however, the relative proportions of the organic acids become similar to those of the mature apple (cf. Turner 1949). There may be several possible explanations of these trends:

(1) The fruit, in the initial period after setting, may resemble a leaf tissue in its enzymic equipment and so produce citric and malic acids in the proportions tending towards those found in apple leaf tissue. It has been found that in apple leaf tissue, the ratio of malic acid to citric acid is approximately 6 : 1; in the first sample in the present experiment the ratio is 20 : 1 but is increased to 80 : 1 by the time of the last sample.

(2) The products of metabolism of the apple leaves may be transported into the developing fruit in approximately the proportion in which they occur in the leaf tissue. In the very young apple, the enzymic capacity is not large enough to cope with the influx of acids but, as development proceeds and the capacity is increased, the relative proportions of the organic acids are gradually transformed to those characteristic of the mature fruit.

(3) They may reflect a drift in the steady state of all constituents arising from the organic acid pool. It is significant in this connection that the changes occur at about the same time as the increase in respiration and the decrease in starch content.

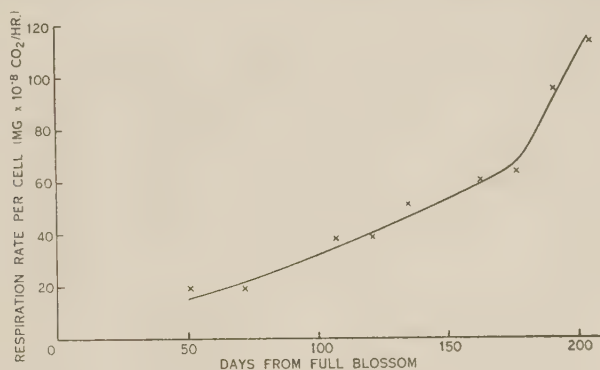


Fig. 5.—Changes in respiration rate per cell of fruits removed after varying periods from full blossom.

(v) *Respiratory Activity*

The respiration rate per cell, shown in Figure 5, which is similar in general form to that expressed on the whole fruit basis, indicates an increase in rate with increasing cell volume. When respiration rate is plotted against cell volume, the curve indicates that the rate of respiration increases more rapidly than cell volume towards the end of the growing period; the ratios of respiration rate to cell volume, given in Table 7, are high for the last two samples.

When the respiration rate per cell is plotted against the protein nitrogen per cell (Fig. 6), the respiration rate in later samples increases more rapidly than the protein nitrogen. Thus, as observed by Hulme and Smith (1938), the linear relationship is observed only over part of the curve; the respiration rate increases more sharply towards the end. This probably represents a climacteric rise at towards the end of the experiment. Earlier work has shown that the climacteric is not particularly well defined in Granny Smith apples. The observation that the respiration rate is high relative to protein nitrogen both at the beginning and at the end of the experiment can be interpreted in several different ways:

- (1) Protein nitrogen synthesis is not strictly dependent on respiration rate.
- (2) Proteins, other than enzyme proteins, may vary from time to time.
- (3) Both protein and enzyme nitrogen vary, but some other factor controls the respiration rate and is responsible for the climacteric rise.

IV. DISCUSSION

From these results, it is possible to obtain a picture of the sequence of increase in cell volume of these fruits and the way these changes are related to chemical changes occurring simultaneously. If growth is to be studied as a phenomenon of cell enlargement, the marked changes in carbohydrate content, i.e. dry weight increasing relatively more rapidly than cell size, make dry weight studies unsuitable as a basis of expression. Fresh fruit weight, which is more closely correlated with fruit size, is more suitable, but even when this is used, the sampling error may seriously interfere with a study of growth regarded as cell enlargement.

TABLE 7
RELATIONSHIP BETWEEN RESPIRATION RATE PER CELL, CELL VOLUME, AND CELL SURFACE FOR SUCCESSIVE PICKS

Pick	Respiration Rate per Cell (mg. $\times 10^{-7}$ CO ₂ /hr.)	Cell Volume (cu. mm.)	Cell Surface (sq. mm.)	Rate per Unit Volume ($\times 10^6$)	Rate per Unit Surface ($\times 10^8$)
1	0.19	0.00047	0.025	53	78
2	0.19	0.0010	0.047	20	41
3	0.38	0.0027	0.093	14	41
4	0.38	0.0026	0.090	15	43
5	0.51	0.0034	0.113	15	45
6	0.60	0.0042	0.125	15	48
7	0.63	0.0044	0.131	14	49
8	0.95	0.0052	0.147	18	65
9	1.14	0.0063	0.168	18	68

As the volume of the cells increased, presumably due mostly to the enlargement of the vacuoles, certain constituents, namely protein nitrogen, soluble nitrogen, total organic acids, and cell wall substance, seemed to increase more with the increase in cell surface than with the increase in cell volume. The linear relationship of protein nitrogen per cell and cell surface suggests that cell expansion was accompanied by synthesis of cytoplasmic proteins, which tended to keep the cytoplasmic layer at approximately constant thickness while the vacuoles of the cells were increasing in volume. This is in agreement with the views first expressed clearly by Frey-Wyssling and Blank (1940) that, in enlarging cells (in their work, in *Zea mais* coleoptiles), synthesis of cytoplasm keeps pace with the enlargement of the cell by vacuolation. Meanwhile, as might be expected, the cell wall material increased in proportion to the increase in cell surface. The fact that soluble nitrogen was so highly correlated with protein nitrogen probably reflects the steady state condition between these two constituents and raises the possibility that the soluble nitrogen supply was the factor which, more than any other, governed the extent of protein synthesis and hence of cell enlargement; the increase in protein nitrogen per cell was maintained throughout the development. It would be interesting to know how much of the soluble nitrogen fraction was in the vacuole and how much was present in the cytoplasmic region.

Meanwhile, during enlargement, the sugar concentration, mostly as sucrose and presumably in the vacuoles, increased throughout the life of the cells.

The rate of respiration per cell increased with time but showed a more rapid increase in the last two samples. It was expected that respiration rate would increase with the protein nitrogen since this increase might represent an increase both in total enzymes, including those of respiration, and in cell constituents, which required a constant transfer of energy for their maintenance. It is probable that the rise in respiration towards the end of the experiment represented a climacteric which, in this variety of apple, might be the result of increased demands for protein maintenance.

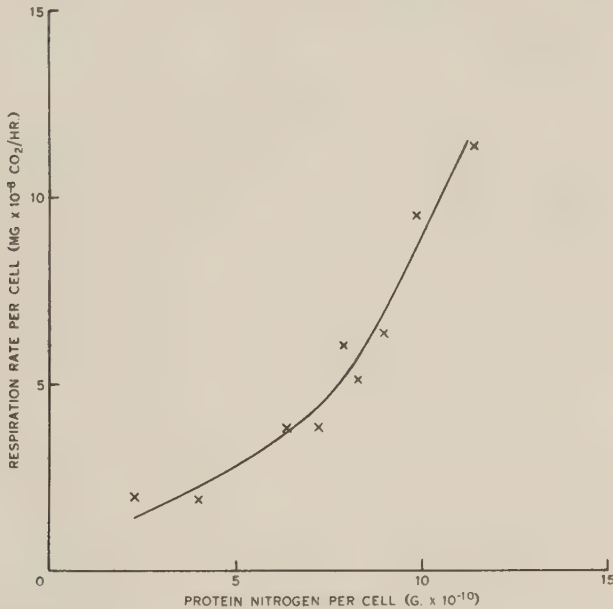


Fig. 6.—Relationship between respiration rate per cell and protein nitrogen per cell.

From analogy with other tissues that have been investigated in detail, it can probably be assumed that energy would be transferred from respiration to the various synthetic processes by the phosphate transfer mechanism. Maskell (1948) has pointed out that starch synthesis in plant tissue probably reflects the level of the effective phosphorylations of carbohydrate, in competition with phosphorylations for other synthetic processes. Further, Arreguin-Lozano and Bonner (1949), in a re-examination of the well-known starch to sugar changes that occur in potatoes at low temperature, have shown that the phosphorylated compounds are affected. This they interpret as being due to an inhibitor of the phosphorylase at higher temperatures that favours the presence of starch. An alternative explanation that would fit their data is that at the higher temperatures, the phosphorylations from respiration are sufficient to keep up an excess of glucose-1-phosphate, which pushes the equilibrium towards starch

synthesis. When this rate of phosphorylation falls with the lower respiration rate at lower temperatures, glucose-1-phosphate no longer occurs in sufficient quantity to maintain the starch level and the other carbohydrates are observed.

The hypothetical relationship suggested by Maskell (1948) between carbohydrate content, protein synthesis (and maintenance), and respiration as connected by the phosphate transfer mechanism offers a tentative interpretation of our observations. It is significant that respiration rate rises steeply as starch content falls; since it is possible that starch may be a measure of the excess energy-rich phosphate production, this fall in starch suggests that other energy-requiring processes are now competing for the energy-rich phosphates. While energy-rich phosphates have been in excess, the respiration rate has been restricted by the absence of phosphate acceptors (i.e. if adenosinediphosphate were the acceptor, its concentration would be low and that of adenosinetriphosphate high), preventing the loss of phosphate from the phosphorylated compounds of the glycolytic and acid cycles. If subsequently, owing to increased demands for phosphorylations in protein syntheses and maintenance, less phosphorylations of sugar, resulting in decrease in glucose-1-phosphate, occur, the equilibrium drifts towards glucose, and consequent decrease in starch. Simultaneously, owing to a more rapid turnover of phosphate carriers with the greater demand in protein synthesis, more phosphate acceptors are available to take energy-rich phosphates from the respiration and an increase in the rate of respiration could be expected. Investigations in progress are examining how far the rise in rate at the climacteric can be explained according to Maskell's hypothesis.

Discussion of the relation of respiration rate to protein level seems to be of limited value until the problem of what controls respiration rate has been solved, but it seems reasonable to postulate that the greater the concentration of unstable protein molecules, the greater will be the transfer of energy-rich phosphate in that direction for maintenance, the quicker will be the dephosphorylations of carriers, and hence the greater will be the respiration rate. Clearly, protein level and respiration rate are inextricably bound up together. This, of course, is a general relation in plants and two principal explanations have been suggested:

(1) The protein content may be an approximate measure of the number of "respiratory seats" (Petrie and Williams 1938), or

(2) The respiration rate may determine the protein level by the mechanisms outlined by Petrie (1943) and Wood (1945). These alternatives are being studied further.

Larger fruit have more cells and, if left on the tree longer, have larger cells. Large fruit may have difficulty in maintaining cell constituents in a system where the higher protein contents are making severe demands on the energy distributors of the cells. These observations may have considerable significance in interpretations of the differences in behaviour of large and small fruit and of fruit from light and heavy crops.

V. ACKNOWLEDGMENTS

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VI. REFERENCES

- ARCHBOLD, H. K. (1932).—*Ann. Bot.* **46**: 407.
ARREGUIN-LOZANO, B., and BONNER, J. (1949).—*Plant Physiol.* **24**: 720.
ASKEW, H. O. (1935).—*J. Pomol.* **13**: 232.
BAIN, J. M., and ROBERTSON, R. N. (1951).—*Aust. J. Sci. Res. B.* **4**: 75.
FREY-WYSSLING, A., and BLANK, F. (1940).—*Nature* **145**: 974.
GUSTAFSON, F. G. (1926).—*Plant Physiol.* **1**: 251.
HACKNEY, F. M. V. (1944).—*Proc. Linn. Soc. N.S.W.* **69**: 91.
HULME, A. C. (1936).—*Biochem. J.* **30**: 258.
HULME, A. C., and SMITH, W. H. (1938).—Report of Food Investigation Board for the year 1938, p. 127.
KIDD, F. (1934).—"The Respiration of Fruits." (Royal Institution of Great Britain: London.)
KIDD, F., and WEST, C. (1945).—*Plant Physiol.* **20**: 467.
KREBS, H. A. (1943).—*Advances Enzymol.* **3**: 191.
KROTKOV, G., and HELSON, V. (1946).—*Canad. J. Res. C.* **24**: 126.
MASKELL, E. J. (1948).—Unpublished Lectures, Cambridge University.
O'GRADY, N. (1942).—M.Sc. Thesis, University of Sydney.
PETRIE, A. H. K. (1943).—*Biol. Rev.* **18**: 105.
PETRIE, A. H. K., and WILLIAMS, R. F. (1938).—*Aust. J. Exp. Biol. Med. Sci.* **16**: 347.
SHAW, S. T. (1942).—*Plant Physiol.* **17**: 80.
TURNER, J. F. (1949).—*Aust. J. Sci. Res. B.* **2**: 138.
WIDDOWSON, E. M. (1932).—*Ann. Bot.* **46**: 597.
WOOD, J. G. (1945).—*Ann. Rev. Biochem.* **14**: 665.

THE EFFECT OF APPLIED PHOSPHATE ON THE UPTAKE OF ZINC BY FLAX

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Summary

An experiment is described in which flax plants were grown in pots in Kojonup gravelly sand containing varying amounts of added phosphate, both with and without added zinc. Dry weight, phosphorus content, and zinc content of the total tops were determined at four periods in the early stages of growth.

Symptoms of zinc deficiency appeared after four weeks in high phosphate treatments. The symptoms were soon apparent in all phosphate treatments and reached a maximum in six weeks, after which recovery of the plants commenced. Marked growth increases due to phosphate and to zinc treatments were apparent.

The degree of severity of the zinc deficiency symptoms and the extent of the response to zinc treatment increased with the level of phosphate application. Analyses showed that, while phosphorus content increased, relative and absolute zinc contents were significantly reduced at all harvests with increasing phosphate application.

In this experiment, the reduction of relative zinc content in the tops of flax plants was sufficient to account for the effect of phosphate application on zinc deficiency symptoms and on response to zinc treatment.

I. INTRODUCTION

Several workers have reported that applied phosphate has induced or accentuated zinc deficiency symptoms in plants. West (1938) described phosphate-induced zinc deficiency in citrus at Griffith. Millikan (1946, 1947), in Victoria, found that excess phosphate, applied in the field as superphosphate and in pots as disodium hydrogen phosphate, induced zinc deficiency symptoms in flax. Cass Smith and Harvey (1948) reported that superphosphate application accentuated zinc dieback in flax in Western Australia. Reuther and Crawford (1948) found that a very heavy application of triple superphosphate to a Californian soil produced foliage symptoms typical of zinc deficiency in grapefruit.

There does not appear to be any evidence that zinc is fixed in the soil by the formation of an insoluble zinc phosphate. On the other hand, there is some evidence that zinc is not fixed by phosphate application. Peech (1941), investigating three light Florida soils, found that, though the phosphorus content varied considerably, they reacted similarly to zinc fixation. In a survey study, Jamison (1943a) produced strong evidence for several Florida soils that phosphate, both naturally occurring and applied as superphosphate, does not cause

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fixation of zinc, except with extreme excesses of both applied phosphate and applied zinc (20,000 lb. per acre of superphosphate containing 6 per cent. water-soluble phosphoric anhydride in conjunction with 750 lb. per acre of zinc sulphate). Where treatments caused changes in pH, effects on the fixation of zinc were noticed, but they were shown to be due to pH changes. Further, he reported (Jamison 1943*b*) that the addition of superphosphate to the soil increased the mobility of zinc owing to the replacing action of calcium and the fact that zinc phosphate is somewhat more soluble than the humate.

It appears that phosphate affects the utilization of zinc by the plant in some other way. Millikan (1940), in a field experiment with wheat, obtained a linear relationship between zinc and phosphorus concentrations in the young plants. He postulated that application of superphosphate increased the "zinc requirement" of plants, and believed that this gave a partial explanation of phosphate-induced zinc deficiency. Over a wider range of zinc and phosphorus concentrations, Piper and Walkley (1943) confirmed the linear zinc-phosphorus relationship for mature oats, but they suggested that zinc impurities in the superphosphate used (Walkley 1940) might have been responsible for the relationship found. In an investigation on the effect of varying rates of application of phosphate (dicalcic phosphate) on the uptake of zinc by oat plants, Rogers and Wu (1948) found in young and mature plants that, as the phosphorus content of the plants increased, zinc content decreased to an almost constant value. Their results strongly support the view that zinc impurities in the superphosphate were responsible for the linear zinc-phosphorus relationship obtained by Millikan (1940) and Piper and Walkley (1943). Furthermore, although they did not discuss their results in the light of phosphate-induced zinc deficiency, the significant decrease in relative zinc content with high phosphate application found by Rogers and Wu (1948) gives a possible explanation of the phosphate-induced zinc deficiency discussed earlier.

The work reported in this paper was undertaken to obtain further information on the effect of phosphate on zinc uptake and its relationship to zinc deficiency in plants. Data for dry weight, phosphorus content, and zinc content of flax plants grown under varying levels of phosphate application are reported for different stages of development.

II. EXPERIMENTAL

(a) *Description of Experiment*

Flax plants (*Linum usitatissimum* var. WADA) were grown in glazed porcelain pots each containing 2 kg. of Kojonup gravelly sand (pH 6.2) obtained from an area that had shown severe zinc deficiency symptoms and marked yield increases with zinc treatment in flax and oat crops. The pots were kept in a glass-roofed bird-cage—the galvanized wire netting surrounding the bird-cage was painted with a bitumen-base enamel to prevent zinc contamination.

Seventy seeds were sown in each pot on May 18, 1950. Germination commenced on May 23 and plants were thinned to 65 per pot seven days later and finally to 55 per pot 12 days after germination.

All pots received the following nutrients (in g. per pot) two days after sowing:

$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.28 g.; KNO_3 , 0.28 g.; K_2SO_4 , 0.14 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07 g.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.009 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.009 g.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.018 g.; $\text{Na}_2\text{B}_4\text{O}_7$, 0.003 g.; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.001 g. On a surface area basis, 0.14 g. per pot = 112 lb. per acre.

All pots were maintained at 60 per cent. water-retaining capacity of the soil by frequent application of water distilled through pyrex glass apparatus.

Six treatments were imposed: phosphorus was applied at three levels with and without zinc. The scheme may be represented:

$$\left. \begin{array}{l} \text{P}_0 \\ \text{P}_1 \\ \text{P}_2 \end{array} \right\} 3 \times \left. \begin{array}{l} \text{Zn}_0 \\ \text{Zn}_1 \end{array} \right\} 2$$

where P_0 = no treatment, P_1 = 0.32 g. per pot $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, P_2 = 0.96 g. per pot $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, Zn_0 = no treatment, Zn_1 = 0.018 g. per pot $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$. On a surface area basis, 0.32 g. per pot $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ = 560 lb. per acre superphosphate (20 per cent. water-soluble P_2O_5). The salts (AR grade) were applied in solution three days after sowing. The pH of the soil was 6.2 and was not affected by treatment.

Harvests were made on the following days after germination:

Harvest I, 11 days, 9 pots; Harvest II, 21 days, 6 pots; Harvest III, 43 days, 3 pots; Harvest IV, 71 days, 3 pots. The number of pots refers to the number per treatment harvested. At each harvest, the plants were cut at the cotyledons and the tops dried overnight in an oven at 105°C ., cooled in a desiccator, and weighed. At Harvests III and IV, the freshly harvested plants were sorted into groups based on the severity of zinc deficiency symptoms. The material was then divided into portions for zinc and phosphorus analyses (care being taken to see that plants from each severity group were equally represented) before drying in the oven. Dry weight data for the total tops are presented in Table 1A. Analysis for variance was used in the statistical analysis.

(b) Analytical Methods

Phosphorus was determined in the tops of the flax plants by the molybdenum blue procedure. At Harvest I, two replicate determinations were made on each treatment. One pot was used for each determination. For all other harvests, three replicate determinations were made on each treatment. At Harvest II, the material from one pot was used for each determination, while at Harvests III and IV, an aliquot from each pot (separated as described above) was used for each determination. The means of the determinations are presented in Table 1D.

Zinc was determined polarographically in a Cambridge Voltamscope using a nitrogen gas chain. The method used was that employed by Walkley (1942). Three replicate determinations were made for each treatment at each

TABLE 1

Harvest Treatment	I			II			III			IV		
	Zn ₀	Zn ₁	Mean	Zn ₀	Zn ₁	Mean	Zn ₀	Zn ₁	Mean	Zn ₀	Zn ₁	Mean
A. Mean dry weight of total tops (g. per pot)	P ₀	0.249	0.243	0.246	0.398	0.394	0.64	0.72	0.68	1.36	1.34	1.35
	P ₁	0.259	0.262	0.261	0.439	0.417	0.97	1.15	1.06	2.71	3.00	2.86
	P ₂	0.243	0.235	0.239	0.427	0.441	1.02	1.28	1.15	2.47	3.30	2.89
	Mean	0.250	0.247		0.421	0.414	0.88	1.05		2.18	2.55	
B. Relative amounts of zinc (p.p.m. dry wt.)	P ₀	64	83	74	33	40	41	45	43	45	52	49
	P ₁	58	72	65	31	38	27	32	30	20	23	22
	P ₂	54	65	60	26	28	24	25	25	18	20	19
	Mean	59	73		30	35	31	34		28	32	
C. Absolute amounts of zinc (µg. per pot)	P ₀	16	20	18	13	16	26	32	29	61	70	66
	P ₁	15	19	17	14	16	26	37	32	54	69	62
	P ₂	13	15	14	11	12	24	32	28	44	66	55
	Mean	15	18		13	15	25	34		53	68	
D. Relative amounts of phosphorus (% dry wt.)	P ₀	0.37	0.34	0.36	0.22	0.23	0.14	0.13	0.14	0.12	0.11	0.12
	P ₁	0.50	0.46	0.48	0.25	0.33	0.41	0.32	0.37	0.21	0.19	0.20
	P ₂	0.90	0.82	0.86	0.53	0.51	0.84	0.64	0.74	0.55	0.40	0.48
	Mean	0.59	0.54		0.33	0.36	0.46	0.36		0.29	0.23	
Level of Sig.												
Analysis of vari- ance—differences required for significance	A All values			1% Level	5% Level	1% Level	5% Level	1% Level	5% Level	1% Level	5% Level	1% Level
	Mean Zn			—	—	—	—	—	—	—	—	—
	Mean P			—	—	—	—	—	—	—	—	—
	B All values			13.5	18.9	9.6	5.1	7.1	5.1	5.1	7.2	—
	Mean Zn			7.8	8.9	—	2.9	4.1	4.1	3.0	—	—
	Mean P			9.5	—	6.8	3.6	5.0	5.0	3.7	5.1	—
	D All values			0.171	0.240	0.200	0.112	0.157	0.157	0.044	0.062	0.062
	Mean Zn			—	—	—	0.065	0.091	0.091	0.026	0.036	0.036
	Mean P			0.121	0.170	0.142	0.079	0.111	0.111	0.031	0.044	0.044

harvest. At Harvest I, two pots were bulked for each determination. At Harvest II, one pot was used for each replicate. At Harvests III and IV, determinations were made on aliquots from each pot (see above). Mean values for relative zinc and statistical analyses are presented in Table 1B.

Absolute zinc was calculated from the means of the dry weight and the means of relative zinc, and the values are presented in Table 1C. In interpreting these data, it must be remembered that, to obtain sufficient material for analysis, 65 plants per pot were harvested at Harvest I and only 55 at succeeding harvests.

III. RESULTS AND DISCUSSION

(a) *Effects of Zinc and Phosphorus on Growth*

At Harvest I, there were no visible effects of zinc on phosphorus treatments, but analysis of the yield data showed that P_2 gave a small significant depression of dry weight relative to P_1 . Williams (1936) obtained a similar reduction of dry weight in oats during adolescence. By Harvest II, no significant differences were apparent from the dry weight data. However, the apices of plants of treatments P_1 and P_2 , were visibly more vigorous than P_0 and, within another two days, growth increases due to phosphorus application were clearly visible ($P_2 > P_1 > P_0$).

On June 19, 28 days after germination, a necrotic spotting of leaves near the crown in some plants of P_2Zn_0 was noticed, and crowns of the plants of this treatment appeared less healthy than those of P_2Zn_1 . The symptoms were suggestive of zinc deficiency. An examination on June 26 showed widespread zinc deficiency symptoms from mild (leaf bronzing, mild rosetting) to severe (severe rosetting, dieback) as described by Millikan (1942, 1946) and Cass Smith and Harvey (1948). The severity of the symptoms was dependent on phosphate treatment. P_2Zn_0 plants were the most severely affected; P_2Zn_1 were less severely affected, while P_1Zn_0 showed symptoms in relatively few plants. There were no symptoms of zinc deficiency in P_1Zn_1 , P_0Zn_0 , or P_0Zn_1 .

The severity of the symptoms continued to increase until the time of Harvest III, when they reached a maximum. At this stage, the mean numbers of affected plants per pot were as follows:

	P_0	P_1	P_2
Zn_0	0	11	34
Zn_1	0	1	17

The dry weight data for this harvest (Table 1A) show no significant effect of zinc at P_0 , a significant increase at P_1 , and a highly significant increase at the P_2 level. Both the relative and absolute effect of zinc on growth increased with each phosphate application. Also, it is obvious that phosphate treatments increased growth. The increase of P_2 over P_1 is not significant, but both treatments show highly significant increases over P_0 . The possible influence of these growth differences on the zinc responses through dilution of zinc is discussed later.

Within a week after Harvest III, some diseased plants showed the typical symptoms of recovery from zinc deficiency by regrowth from rosetted apices or from the axils of the cotyledons. By Harvest IV all diseased plants showed signs of recovery but yield data revealed that responses to zinc and phosphorus were of the same order as in the previous harvest. With the significant depression in growth of P_2Zn_0 relative to P_1Zn_0 the effect of increasing phosphate application on response to zinc was even more obvious than at Harvest III.

From the evidence presented here it is clear that application of sodium dihydrogen phosphate to Kojonup gravelly sand induced zinc deficiency symptoms in flax plants and produced marked responses to zinc treatment. The severity of the symptoms and the relative response to zinc increased with increasing phosphate application.

(b) Zinc and Phosphorus Analyses

The relative amount of phosphorus in the tops of the flax plants increased with increasing phosphate application at all harvests. In Harvests III and IV the differences in relative phosphorus content between zinc treatments may be explained by the promotion of growth by zinc application resulting in dilution of phosphorus.

The relative amount of zinc in the tops was depressed at every harvest by phosphate application. The depression of relative zinc content at Harvests III and IV may be partly accounted for by dilution of zinc through more rapid growth of phosphate treatments. But it is obvious that some factor other than dilution must have caused the significant depression of relative zinc content at Harvests I and II. Furthermore, the absolute amount of zinc in the tops was depressed at all harvests by phosphate application.

The data for relative zinc content confirm the results of Rogers and Wu (1948) discussed earlier. There is no evidence that high phosphorus content interferes with utilization of zinc in the tops of flax plants as suggested by Millikan (1940). However, the depression of relative zinc content in the tops of flax plants by the application of phosphate provides an adequate explanation for the effect of phosphate on growth responses to zinc treatment and on the incidence of zinc deficiency symptoms. This depression cannot be entirely accounted for by dilution effects resulting from growth responses; applied phosphate apparently exercises a direct effect on either the uptake of zinc by the roots, or the movement of zinc (Wood and Sibly 1950) from the roots to the tops of flax plants.

IV. ACKNOWLEDGMENTS

Grateful acknowledgment is made to the Commonwealth Research Grants to Universities for financial assistance and to Mr. R. C. Rossiter, Division of Plant Industry, C.S.I.R.O., and Dr. T. C. Dunne of the Western Australian Department of Agriculture for help and advice.

V. REFERENCES

- CASS SMITH, W. P., and HARVEY, H. L. (1948).—*J. Dep. Agric. W.A.* **25**: 136-42.
- JAMISON, V. C. (1943*a*).—*Proc. Fla. Sta. Hort. Soc.* **56**: 26-31.
- JAMISON, V. C. (1943*b*).—*Soil Sci. Amer. Proc.* **8**: 323-6.
- MILLIKAN, C. R. (1940).—*J. Dep. Agric. Vict.* **38**: 135-6.
- MILLIKAN, C. R. (1942).—*J. Aust. Inst. Agric. Sci.* **8**: 33-5.
- MILLIKAN, C. R. (1946).—*J. Dep. Agric. Vict.* **44**: 69-73.
- MILLIKAN, C. R. (1947).—*J. Dep. Agric. Vict.* **45**: 273-8.
- PEECH, M. (1941).—*Soil Sci.* **51**: 473-86.
- PIPER, C. S., and WALKLEY, A. W. (1943).—*J. Coun. Sci. Industr. Res. Aust.* **16**: 217-34.
- REUTHER, W., and CRAWFORD, C. L. (1948).—*Soil Sci.* **62**: 477-91.
- ROGERS, L. H., and WU, CHIH HWA (1948).—*J. Amer. Soc. Agron.* **40**: 563-6.
- WALKLEY, A. (1940).—*J. Coun. Sci. Industr. Res. Aust.* **13**: 255-60.
- WALKLEY, A. (1942).—*Aust. J. Exp. Biol. Med. Sci.* **20**: 139-47.
- WEST, E. S. (1938).—*J. Coun. Sci. Industr. Res. Aust.* **11**: 182-4.
- WILLIAMS, R. F. (1936).—*Aust. J. Exp. Biol. Med. Sci.* **14**: 165-80.
- WOOD, J. G., and SIBLY, PAMELA M. (1950).—*Aust. J. Sci. Res. B* **3**: 14-27.

THE TRANSMISSION OF WITCHES' BROOM VIRUS DISEASE OF LUCERNE BY THE COMMON BROWN LEAFHOPPER, *OROSIUS ARGENTATUS* (EVANS)

By G. A. H. HELSON*

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Summary

The disease was transmitted by *Orosius argentatus* (Evans) collected in infected lucerne crops, and also by groups of virus-free individuals reared under experimental conditions, after having been fed on plants infected with witches' broom virus.

The following plants became infected with the virus under experimental conditions: *Beta vulgaris* L.; *Datura stramonium* L., *Erodium cicutarium* (L.) L.Hérit, *Hypochaeris radicata* L., *Lycopersicon esculentum* Mill., *Medicago sativum* L., *Vinca rosea* L. Lucerne was difficult to infect and the first symptoms took about seven months to appear (but see note on infection of lucerne).

In all plants the symptoms caused were severe stunting, proliferation of axillary shoots, and the production of green flowers. In tomato and other hosts these symptoms closely resembled those of tomato big bud virus. The same vector transmits them and it is suggested that the two diseases may be caused by the same or closely related strains of virus.

I. INTRODUCTION

Witches' broom, a virus disease of lucerne (*Medicago sativum* L.), is prevalent throughout the inland areas of Queensland, New South Wales, Victoria, and South Australia and has been reported from north-western Australia and the west coast of the United States of America (Menzies 1946). The disease is characterized by severe stunting and proliferation of shoots produced from the crown (Plate 1, Fig. 1). Usually, plants fail to flower, but occasionally very small blooms or green flowers are produced (Plate 1, Fig. 2). The disease has been in Australia for at least 40-45 years and was first recognized to be caused by a virus by Edwards (1935*a*, 1935*b*, and 1936) who was able to transmit it to healthy lucerne plants by grafting. Field surveys made by Edwards demonstrated that lucerne fields commonly showed 20-25 per cent. infection with the disease and occasionally old stands were observed in which 60-70 per cent. infection occurred. Diseased plants yielded 37 per cent. less weight of green fodder than was obtained from unaffected plants (Edwards 1935). In addition, the disease considerably shortened the life of lucerne stands in regions receiving an annual rainfall of 17-21 in. and, where grown for seed production, the diseased plants were a total loss (Edwards 1936). Edwards

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considered it highly probable that the disease was spread, under natural conditions, by an insect vector but his attempts to transmit the disease with insects were unsuccessful.

The present paper describes the results of the first year of an ecological survey of leafhoppers occurring in infected lucerne fields along the Lachlan River, N.S.W., and in the Australian Capital Territory, and the results of transmission experiments with one of these, *Orosius argentatus* (Evans).

II. FIELD SURVEY

Before experiments for the transmission of the disease could begin it was necessary to know what possible insect vectors frequented fields of lucerne where the disease was prevalent. As the disease was suspected of belonging to that group of virus diseases, most of which are transmitted by leafhoppers, it was decided to concentrate on these insects and to make collections from eight stations, along the River Lachlan between Cowra and Jemalong, N.S.W., at three-monthly intervals. In addition, weekly sweeps were made at Dickson Experiment Station, A.C.T., on a lucerne field infected with witches' broom.

Where possible, on the field survey, sweeps were made with two 14-inch nets trawled over the lucerne from the sides of a truck moving at 10 miles per hour over a distance of one-fifth of a mile. Four such sweeps were made at each station. Where it was not possible to sweep from the truck, two hundred hand sweeps were made. At Dickson Experiment Station, all sweeps were made with two 14-inch nets from the sides of a jeep travelling at 10 miles per hour. Two traverses of 0.1 mile each were made across each diagonal of the field and the total number of insects caught in the four traverses was recorded.

Possible vectors included in the large numbers of insects caught were aphids, thrips, and leafhoppers and of these leafhoppers were the most abundant. During the 1947-48 survey the following jassids commonly occurred in lucerne fields at all eight stations on the Lachlan River and at Dickson Experiment Station: *Orosius argentatus* (Evans), *Erythroneura ix* Myers, *Empoasca viridigrisea* Paoli, *Balclutha* sp., *Eurinoscopus punctatus* Ev., *Nehela torrida* Ev., *Exitianus (Euscelis) indicus* (Dist.) (= *Euscelis norrisi* Ev.), *Nesoclutha obscura* Ev., *Euscelis* sp., and *Thamnotettix* sp. The first three were the most abundant at all places.

At Dickson, A.C.T. (Fig. 1), the weekly sweeps began on November 6, 1947. *O. argentatus* reached its maximum numbers by mid November, and except for a short period in December when heavy rains and cold weather prevailed, the number remained at a high level until the third week in December. During this period, *E. ix*, *E. viridigrisea*, *E. punctatus*, and *Balclutha* sp. were present in small numbers only. After the end of December, the number of *O. argentatus* decreased, whereas the other four leafhoppers all increased in numbers, but at no stage did they approach the maximum recorded for *O. argentatus* during the early summer. This species had a small autumn maximum in late February and March. By the end of May all species (except *E. ix*, small numbers of which occurred throughout the winter) ceased to be taken

in the sweeps and did not reappear until spring. *O. argentatus* began to appear in the sweeps again in the second week in September and reached a spring peak by early November. This peak, however, was only one-third as great as that of 1947. *O. argentatus* was therefore most abundant during spring and early summer whereas the other four species were most abundant during summer and autumn.

Of these species, *O. argentatus* was the only leafhopper known to be a vector of virus diseases. It transmits tobacco yellow leaf dwarf and tomato big bud virus diseases and was therefore chosen for the initial witches' broom transmission experiments in the glass-house. Also, it belongs to the sub-family Euscelinae, which contains a large number of vectors of virus diseases, including *Circulifer tenellus* (Baker), which transmits curly-top of sugar beet in California (Shaw 1910), and *Eutettix phycitis*, which transmits "little-leaf" of egg-plant in India (Thomas 1939).

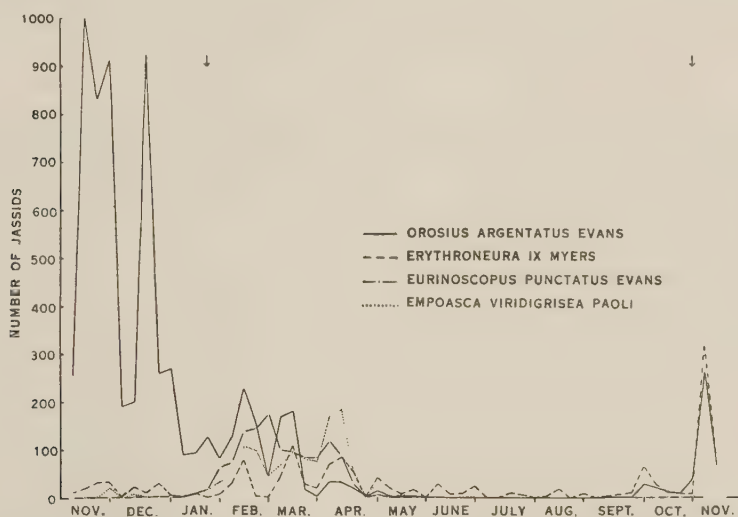


Fig. 1.—Seasonal abundance of jassids on lucerne at Dickson Experiment Station, A.C.T., 1947-48. The arrows indicate flights.

III. GEOGRAPHICAL DISTRIBUTION OF THE VECTOR

Orosius argentatus is widely distributed throughout Australia and extends from the moist tropics in the north to the dry temperate regions in the south and west. It feeds on a large number of host plants and is able to breed on many of these (Helson 1942). It also appears to be widely distributed throughout the Australian region, for Oman (personal communication) records having seen specimens from Eniwetok, Ulithi, Guam, Larat, Amboina, Fiji (collected 1908), and Canton I. (Oman 1943) in the Australian region and specimens that are probably *argentatus* from Sunday I. in the Kermadec Group, New Zealand region, and from the Philippines in the oriental region. To date this leafhopper has not been recorded from the Hawaiian Is. or from New Zealand.

IV. BIONOMICS OF VECTOR

The habits and life cycle of *O. argentatus* have been recorded elsewhere (Helson 1942). In southern inland districts, there are three complete but overlapping generations in a year. The length of the life cycle varies from 32 to 50 days, depending on the time of the year. Breeding plants in addition to those already listed (Helson 1942) are: *Brassica adpressa* Boiss. (hairy brassica), *Silybum marianum* (L.) Gaertn. (variegated thistle), *Sonchus oleraceus* L. (common sow thistle), *Plantago lanceolatum* L. (ribwort), *Callistephus chinensis* (L.) Nees (aster), and *Atriplex semibaccata* R.Br. (creeping saltbush).

Aster is a favoured host plant, both for feeding and breeding, whereas tomato, tobacco, and lucerne are not preferred. In the laboratory, the leafhopper not only fails to breed on these but rapidly dies if fed exclusively on them. However, the insects thrive in the lucerne fields where other feeding plants are generally present.

V. TRANSMISSION EXPERIMENTS

(a) *By Naturally Infected Leafhoppers*

In November 1947, numbers of wild *O. argentatus* were collected from a lucerne field at Dickson Experiment Station, by the method already described, and were placed in a cage in the glass-house with a plant of *Datura stramonium* and a small-flowered malva (*Malva parviflora*). Thirty-five days later the datura* leaves showed vein banding and interveinal chlorosis, and 62 days later green flowers were produced, the leaves were reduced in length and width, and axillary growth was stimulated. The malva did not develop any virus symptoms.

In December 1947, a second field collection of leafhoppers was made in the same way and these also were placed in a cage in the glass-house together with four datura plants and three malva plants. After a period of 44 days, one of the daturas produced green flowers and had vein banding on the young leaves, one had definite vein banding on the young leaves, and the other two plants showed interveinal chlorosis of the young leaves. Seventy-four days after being placed in the cage with leafhoppers, all datura plants were producing green flowers. The malva plants did not exhibit any symptoms of virus disease. Control plants used as food plants for the virus-free insect colonies were all flowering normally at this time.

A scion, taken from one of the daturas with the green flowers, was then grafted to a young tobacco seedling (variety Hickory Pryor) 156 days after the datura had been exposed to the insects. The graft was successful and within 36 days the veins on the young tobacco leaves began to whiten and the leaf blades took on a glazed appearance. One hundred and fifty-six days after grafting, the plant had produced green flowers and was producing small bunched terminal leaves, both pronounced symptoms of virus disease (Plate 2, Fig. 1).

As these first tests were made with wild insects collected from a field of lucerne, the virus disease transmitted to daturas in the two experiments just

*Unless otherwise stated, "datura" = *Datura stramonium*.

described could have been acquired from weeds or other crop plants growing in the vicinity at that time.

(b) *By Laboratory-Infected Leafhoppers*

A series of laboratory experiments using virus-free insects from colonies established in the glass-house were conducted to show that the disease obtained from lucerne in the laboratory was the same as that carried by the leafhoppers collected in the field. Virus-free insects were reared from parents that had been picked off their breeding plants as they hatched and transferred to virus-free breeding plants before they had commenced feeding. No symptoms developed in these plants nor in other healthy daturas added to the colonies from time to time to test the virus-free insects.

A lucerne plant infected with witches' broom virus disease taken from the lucerne field in 1946 was used as the sole source of virus disease in all the later experiments (Plate 1, Fig. 1). The experimental results appear in Table 1.

TABLE 1
HOST PLANTS TO WHICH WITCHES' BROOM VIRUS DISEASE OF LUCERNE WAS
TRANSMITTED BY THE COMMON BROWN LEAFHOPPER, *OROSIUS ARGENTATUS* (EV.)

Host Plant	Number of Insects	Time in Days			
		Fed on Infected Lucerne	Fed on Healthy Host	To Appearance of Symptoms	To Appearance of Green Flowers
<i>Medicago sativa</i>	8	7	14	195	—
<i>Datura stramonium</i>	44	10	96	81	96
<i>Datura stramonium</i>	44	10	83	—	83
<i>Datura stramonium</i>	44	10	83	—	83
<i>Datura stramonium</i>	44	10	15	—	44
<i>Lycopersicon esculentum</i>	44	10	35	35	44
(var. Rouge de Marmonde)					
<i>Lycopersicon esculentum</i>	44	10	35	35	47
(var. Rouge de Marmonde)					
<i>Hypochaeris radicata</i>	44	10	15	41	—
<i>Erodium cicutarium</i>	44	10	15	41	48
<i>Vinca rosea</i>	92	14	76	68	68
<i>Beta vulgaris</i>	100	20	20	123	—
<i>Beta vulgaris</i>	10	3	14	129	—

Experiment 1.—Forty-four laboratory-infected *O. argentatus* were placed in a cage with one datura, one malva, and one lucerne plant. Eighty-one days after first exposure to the insects, the datura began to show vein banding and interveinal chlorosis of the leaves, and 96 days from the beginning of the experiment was producing green flowers. The datura was still showing pronounced virus symptoms and was producing green flowers (Plate 2, Fig. 2) 146 days after exposure. The malva died without showing any symptoms, and the lucerne plant remained healthy.

Experiment 2.—Two datura plants added to the same cage produced green flowers in 83 days and showed pronounced symptoms of virus disease after 112 days. Another datura was then added to the cage for 15 days, after which it was taken out and pruned. Forty-four days after pruning it too produced green flowers, and 62 days later was showing pronounced virus symptoms. Three other daturas and one *D. tatula* used as feeding and breeding plants in the cage likewise produced green flowers and other pronounced virus symptoms.

Experiment 3.—Two tomato seedlings were placed for 35 days in the cage containing the infected plants on which the leafhoppers were then breeding. At the end of this time, the veins of the leaves were beginning to appear translucent so the plants were removed and kept for observation. At the end of 44 days, the main veins of the young leaves and the growing tips were showing a pronounced purpling, the marginal leaflets were showing epinasty, and the leaves were reduced in size. The veins were translucent and prominent, giving a netted appearance. One plant had already produced green flowers and the second plant did so three days later. Fifty-six days from the beginning of the experiment, the plants were showing very definite signs of virus disease. These became more and more pronounced as time went on and the axillary buds of both plants grew and produced dwarf leaves with purple veins with a curling and twisting of the main vein. The leaves were rugose and at 89 days the plant showed a rosetted appearance at the top caused by the stunting of growth of the main stem and the proliferation of numerous axillary shoots with dwarf leaves and green flowers. One month later the plants were still dwarfed and remained so until death (Plate 3, Fig. 1).

Experiment 4.—One common crowfoot plant (*Erodium cicutarium*) was added to the same cage one month after the two tomato seedlings and after 15 days was removed and kept for observation. Forty-one days later, the older leaves of the plant were curled and rolled downwards and the new leaves were a light green, erect, and spindly. A week later the young leaves were light green, very much reduced in size, with vein banding, and the plant produced small, green flowers on thin, spindly stems. Similar symptoms were observed in the field locally (Plate 3, Fig. 2); 89 days after the experiment began, the plant was dying.

Experiment 5.—One month after the two tomato seedlings, a flatweed plant (*Hypochaeris radicata*) was also added to the same cage and allowed to remain for 15 days. Odd spindly leaves began to appear 41 days later and the new leaves were chlorotic. This was the same as the time taken for virus symptoms to appear in the crowfoot. One week later the plant had produced a large number of new leaves, much reduced in size, spindly, and with narrow, chlorotic leaf blades. The plant collapsed and died from a crown necrosis 54 days after the experiment began and before flowers were produced, so it is not known whether green flowers would have been formed. Plants with green flowers but no leaf symptoms were subsequently found in the field.

Experiment 6.—One hundred virus-free *O. argentatus* were allowed to feed for 20 days on two daturas, three sugar beet (*Beta vulgaris*), one malva, and the diseased lucerne in a cage. The plants were then removed and kept for observation. One of the three sugar beet plants began to throw spindly leaves with very narrow leaf blades 123 days later, and within 183 days was showing pronounced virus symptoms. The plant wilted and collapsed 219 days after the beginning of the experiment, and showed a crown necrosis very similar to that of the flatweed. Again no flowers were formed before death. All the other plants remained healthy. In a second experiment with a sugar beet on which 10 infective insects fed for three days, the plant became similarly affected in 129 days (Plate 4).

Experiment 7.—A *Vinca rosea* seedling was placed in the transmission cage for 76 days with 92 leafhoppers that had fed on the witches' broom-infected lucerne. Forty-eight days after exposure commenced, leafhoppers were still feeding on the plant, which was flowering normally (white flowers) and resembled its control plant in every respect. Sixty-eight days from the beginning of the experiment, however, green flowers were produced and thereafter a few green and white flowers were formed, together with some entirely green flowers in which the corolla tube was shortened. In some instances the gynoeceium became swollen and burst through the walls of the corolla. Thereafter the plant continued to produce green flowers.

Experiment 8.—As the disease had not so far been transmitted from lucerne to lucerne, 80 virus-free jassids were confined on the diseased lucerne plant for seven days. However, lucerne is not a preferred host plant and the leafhopper cannot breed upon it (Helson 1942). A high mortality always results where these insects are placed on lucerne for more than one or two days without an alternate host plant. Only eight leafhoppers were recovered at the end of the feeding time and these were placed in a cage with two healthy lucerne seedlings for 14 days. All the leafhoppers died by the end of this period, and the plants were retained for observation. One plant subsequently died, but at the end of 195 days, the other began producing small, light green rugose leaves more rounded than usual. This rugosity and roundness, apparently symptoms of witches' broom, became more pronounced at 215 and 250 days.

VI. DISCUSSION

All the plants infected with witches' broom virus developed similar symptoms, namely stunting of growth, proliferation of axillary buds and, most characteristic of all, production of green flowers, frequently after normal flowering. Green flowers have also been observed on lucerne in the field. In the autumn of 1948 these were fairly common on plants showing an advanced stage of the disease in an old stand at Canowindra and at Dickson, A.C.T. (Plate 1, Fig. 2).

The production of green flowers and the stunting of growth, on hosts other than lucerne, is so consistent and characteristic that it bears a striking

similarity to the effect of tomato big bud on its various hosts (Samuel, Bald, and Eardley 1933; Simmonds 1936; Hill 1943). Big bud is also transmitted by *O. argentatus* (Hill 1943) and on tomato, under green-house conditions, may produce very similar symptoms to witches' broom (Plate 5; cf. Plate 3, Fig. 1). Witches' broom of lucerne, when transmitted to tomato, causes a proliferation of the internal phloem as observed by Samuel, Bald, and Eardley (1933) in big bud. The symptoms of the two diseases on *D. stramonium*, tobacco, sugar beet, and crowfoot are also similar. For these reasons it is considered possible that the two diseases may be caused by the same virus or by strains of it. Further evidence is required before this can be said with certainty but the information already obtained justifies further investigation.

If it is true that the two diseases are caused by the same, or by strains of the same, virus, then the relationship between the incidence of big bud on tomato and the seasonal abundance of the vector at Dickson, A.C.T., in 1947-48 is of interest. As has already been pointed out, the leafhopper reached its greatest numbers early in November and the population remained at a high level until the end of December. The percentage of tomatoes in this area that became infected with big bud increased about the middle of February, following swarming of the leafhoppers in the last week of January 1948 (Fig. 2).

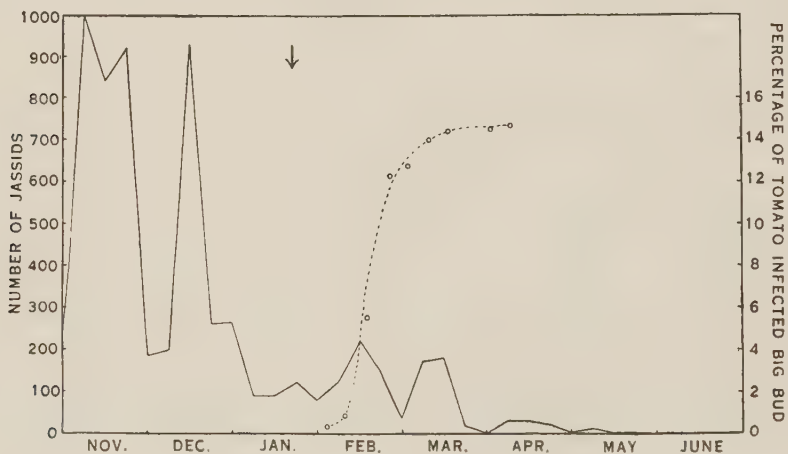


Fig. 2.—Seasonal abundance of *Orosius argentatus* (Evans) on lucerne, and incidence of big bud on tomato (broken line) at Dickson Experiment Station, A.C.T., 1947-48. The arrow indicates a flight.

The geographical distribution of diseases characterized by greening and proliferation is also very interesting. Such a disease has been reported on tobacco, egg plant, black nightshade, and thorn apple in Rumania (Ghimpu 1931). Fruit woodiness or stolbur on tomato has been reported by Richkow, Karatschevsky, and Michailova (1934), in the U.S.S.R., and Rykoff (1935) considered that the same disease infected tobacco, tomato, chilli, *Convolvulus arvensis*, *Atropa belladonna*, *Datura* spp., and other members of the Solanaceae. The changes in the flowers of tomatoes observed by Kostoff (1933) in Russia

WITCHES' BROOM DISEASE OF LUCERNE



Fig. 1



Fig. 2

WITCHES' BROOM DISEASE OF LUCERNE



Fig. 1



Fig. 2



Fig. 1



Fig. 2

WITCHES' BROOM DISEASE OF LUCERNE



WITCHES' BROOM DISEASE OF LUCERNE



agreed very closely with those described by Samuel, Bald, and Eardley (1933) for big bud on tomato in Australia. Ryjkoff therefore concluded the two diseases were caused by the same virus. The disease in Russia is transmitted by *Hyalesthes obsoletus* (Cixiidae, Sukhov 1948). In India, a closely related disease, "little-leaf" of egg plant, is transmitted by the leafhopper, *Eutettix phycitis* (Thomas and Krisnaswami 1939). Greening of *Crotalaria usaramoensis* has been observed in Java but its method of transmission is not known (J. van der Vecht, personal communication). In the Australian region, a similar disease has been reported on tomatoes, antirrhinum, zinnia, petunia, and false cape gooseberry from Suva (Parham, personal communication). Big bud of tomato has been reported from the Pacific north-west of the U.S.A. (Dana 1940), transmission in this case being effected by graft only. A similar disease on lucerne in the Pacific north-west is considered by Edwards (1935a) to be the same disease as witches' broom of lucerne in Australia. It is transmitted by another leafhopper, *Platymoides acutus* (Menzies 1946). To date no similar disease has been recorded from New Zealand or the Hawaiian Islands.

Thus it will be seen that a group of very similar virus diseases occur from Europe across Asia through the Netherlands East Indies to Australia and extend across the Pacific to the West Coast of the U.S.A. There is no evidence that the diseases are, in fact, related but further investigations should prove interesting and profitable.

VII. ACKNOWLEDGMENTS

Many of the results of this investigation would not have been possible without the ready cooperation of lucerne growers in the Lachlan Valley, New South Wales. Grateful acknowledgment is made for the assistance rendered by Messrs. Weir Brothers, Mr. A. Sergeant, and Mr. W. C. Goodacre, on whose properties the ecological surveys were made; by Dr. J. W. Evans and Dr. P. W. Oman in the identification of leafhoppers; by Mr. N. Grylls, who assisted with field surveys; and by Mr. R. Brock, who supplied the figures on the incidence of big bud.

VIII. REFERENCES

- DANA, B. F. (1940).—Occurrence of big bud of tomato in the Pacific north-west. *Phytopathology* **30**: 866-9.
- EDWARDS, E. G. (1935a).—Witches' broom. A new virus disease of lucerne. *J. Aust. Inst. Agric. Sci.* **1**: 31-2.
- EDWARDS, E. G. (1935b).—The witches' broom disease of lucerne. *Rep. Aust. Ass. Adv. Sci.* **323-4**.
- EDWARDS, E. G. (1936).—The witches' broom disease of lucerne. N.S.W. Dep. Agric. Sci. Bull. No. 52.
- CHIMPU, V. (1931).—Contribution à la teratologie des *Nicotiana*. *Rev. Path. Vég.* **18**: 289-95.
- HELSON, G. A. H. (1942).—The leaf-hopper *Thamnotettix argentatus* Evans, a vector of tobacco yellow dwarf. *J. Coun. Sci. Industr. Res. Aust.* **15** (2): 175-84.
- HILL, A. V. (1943).—Insect transmission and host plants of virescence (big bud of tomato). *J. Coun. Sci. Industr. Res. Aust.* **16** (2): 85-91.
- KOSTOFF, D. (1933).—Virus diseases causing sterility (Eng. title). Abstract in *Rev. Appl. Mycol.* **12**: 599.

- MENZIES, J. D. (1946).—Witches' broom of alfalfa in North America. *Phytopathology* 36 (9): 762-74.
- OMAN, P. W. (1943).—A new leafhopper from Oceania. *Pan-Pacif. Ent.* 19 (1): 33-4.
- RICHKOW, V., KARATSCHEVSKY, J., and MICHAİLOVA, P. V. (1934).—On the woodiness of fruit in tomatoes. Preliminary note (Eng. title). Abstract in *Rev. Appl. Mycol.* 13: 133.
- RYJKOFF, V. L. (1935).—Filterable virus as a cause of virescence of flowers (Eng. title). Abstract in *Rev. Appl. Mycol.* 14: 130-1.
- SAMUEL, G., BALD, J. G., and EARDLEY, C. M. (1933).—"Big bud," a virus disease of the tomato. *Phytopathology* 23: 641-53.
- SIMMONDS, J. H. (1936).—Disease of the tomato. *Qd. Agric. J.* 45: 5-11.
- SUKHOV, K. S., and VOVK, A. M. (1948).—Woodiness of Solanaceae and the means of combating it (Eng. title). Abstract in *Rev. Appl. Mycol.* 27: 48.
- SHAW, H. B. (1910).—The curly top of sugar beet. U.S.D.A. Bur. Plant Industr. Bull. No. 181.
- THOMAS, K. M., and KRISNASWAMI, C. S. (1939).—Little leaf—a transmissible disease of bringal. *Proc. Ind. Acad. Sci.* 10: 201-12.

EXPLANATION OF PLATES 1-5

PLATE 1

- Fig. 1.—Lucerne plant affected with witches' broom in an advanced stage of the disease. Healthy plant on right.
- Fig. 2.—Lucerne shoots from field affected with witches' broom showing green flowers (arrows).

PLATE 2

- Fig. 1.—*Nicotiana tabacum* showing witches' broom transmitted by graft 156 days after grafting scion *Datura stramonium* to which the disease was transmitted by *Orosius argentatus*. Healthy plant on left.
- Fig. 2.—*D. stramonium* plant infected with witches' broom 146 days after exposure to infective *O. argentatus*. Healthy plant on left.

PLATE 3

- Fig. 1.—Tomato plants (var. Rouge de Marmonde) with symptoms of witches' broom 124 days after exposure to *O. argentatus*, showing rosette appearance. The diseased plant did not grow any further and began to die 31 days later. Healthy plant on left.
- Fig. 2.—*E. cicutarium* collected in field, Canberra, A.C.T., naturally infected with virescence. Healthy shoot on left, shoot showing dwarfing and green flower in centre, and shoot with green flowers and incipient seed pods on right.

PLATE 4

- Beta vulgaris* showing plant in foreground affected by witches' broom 183 days after exposure to infective *O. argentatus*. Plants in background remained healthy.

PLATE 5

- Tomato plant showing big bud symptoms.

A STUDY OF SOME ASPECTS OF THE FEEDING OF THE JASSID *OROSIUS*

By M. F. DAY* and ANNE MCKINNON*

[Manuscript received November 13, 1950]

Summary

The jassid *Orosius* ingested radiophosphorus when this isotope was incorporated into a plant. The insect excreted approximately 65 per cent. of the ingested ^{32}P within 30 minutes after feeding had begun. The amount ingested plus the amount excreted increased linearly with time for the duration of the experiments (up to 3 days). There was considerable variation in uptake between individual jassids. No evidence for transmission of radiophosphorus to another plant was detected. Anaesthesia by carbon dioxide had little or no effect on subsequent feeding. Starvation for about 30 minutes before feeding slightly increased the amount ingested in a 30-minute feeding period. Hydrogen ion or sugar concentration of a liquid artificial diet did not significantly alter the amount ingested.

I. INTRODUCTION

The jassid *Orosius argentatus* (Evans) is a vector in Australia of at least two economically important virus diseases, tobacco yellow dwarf (Hill 1941) and witches' broom of lucerne (Helson 1951). In a series of experiments designed to determine the relationship of the vector to the second disease, transmission was found to be somewhat irregular (Grylls, unpublished data). This irregularity had been reported in earlier work with other jassids (cf. Storey 1939), and so it was desirable to determine whether these differences were due to variations in feeding. It was also required to determine whether the feeding was modified by some of the procedures routinely used in handling the insects, among them being the effects of carbon dioxide and of various periods of starvation. It seemed likely that these problems would be readily tackled by incorporating radiophosphorus in plants or in an artificial diet, and experiments on these points are reported below. No previous attempt to study these aspects of the feeding of jassids has been published, although a somewhat similar method was used by Hamilton (1935) on the aphid *Myzus persicae* with radium as the tracer, and by Carter (1945) on the coccid *Pseudococcus* using ^{32}P incorporated in agar blocks.

II. METHODS

Radiophosphorus as $\text{NaH}_2^{32}\text{PO}_4$ was obtained from the British Atomic Energy Research Establishment and assayed in a lead castle with a commercial scale-of-8 Geiger counter circuit using a GM tube with a "duralumin" window

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of 6 mg. per sq. cm. thickness. Two types of experiment were performed. In the first, tracer doses of the isotope were added to Knop's solution into which the washed roots of entire young plants of the beet *Beta vulgaris* L. were placed for about 24 hours. The roots were then washed and kept in normal Knop's solution for the duration of the experiment. They remained in a healthy condition for the period for which they were required, usually about a week. *Datura stramonium* L., also a foodplant of *Orosius*, proved less useful than *Beta* in these experiments.

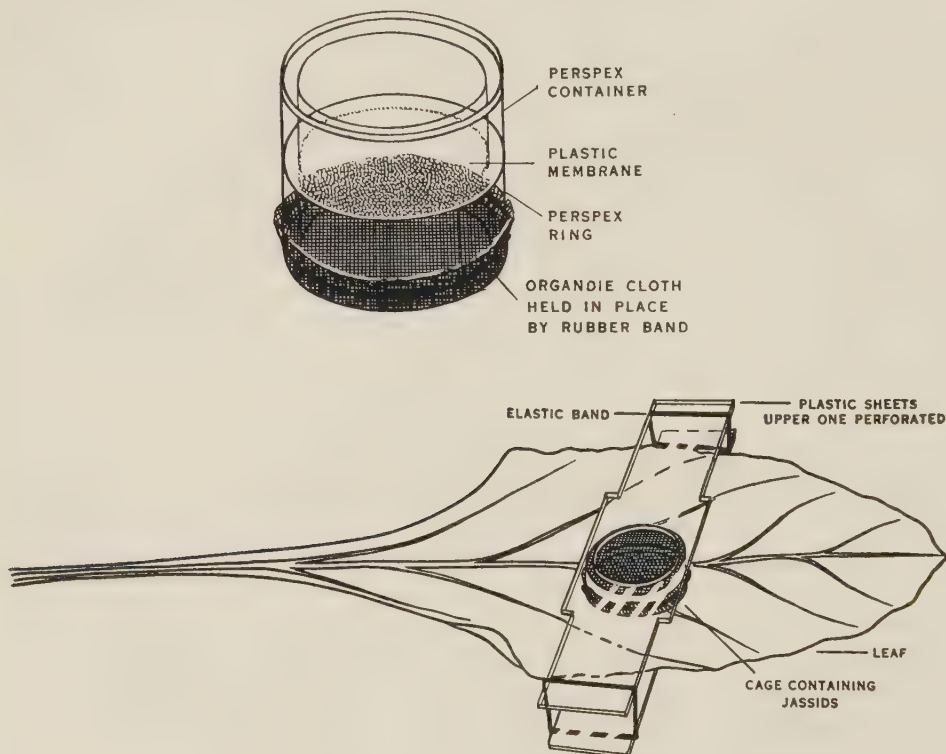


Fig. 1

Above.—Method used for feeding *Orosius* on sugar solutions.

Below.—Method used for feeding *Orosius* on beet leaves

The second group of experiments involved feeding the insects on an artificial diet of sugars (either 1.6 per cent. sucrose or Carter's (1928) medium consisting of 0.2 g. dextrose, 0.5 g. raffinose, 1 g. maltose, 1 g. sucrose, and 1 g. lactose in 250 ml. distilled water) in which were incorporated tracer quantities of ^{32}P . The jassids fed on this liquid through a film of commercial plastic material* arranged as in Figure 1.

The insects were from stocks of a virus-free colony maintained in the glass-house. In the plant experiments they were placed in glass rings of about $\frac{3}{4}$ in. diameter and $\frac{1}{4}$ in. height to which plastic screen was attached by adhesive

* "Plas-B-Loon," supplied by Lonsdale Distributors Pty. Ltd., Melbourne.

on one side and muslin by a rubber band on the other. Usually there were five insects in each cage. The cages were set on the beet leaves and held in place by plastic sheets and rubber bands as illustrated in Figure 1. The insects fed readily through the muslin. Cages containing no jassids took up no ^{32}P from the leaves by contact. For measurement of the uptake of ^{32}P the cage was simply removed from the leaf and deposited the appropriate distance from the counting tube.

The ^{32}P activity was measured in a series of nine insects both by this method and by digestion in nitric acid and subsequent neutralization and transference to standard aluminium sample dishes. The results obtained by the latter method were higher than those from the living insects, but the difference could be accounted for by the higher backscatter, from the improved geometrical relationship of the samples, and by the reduction in internal absorption. Because of the very good correlation between the two series of assays the remainder of the experiments were performed without digestion of the tissues. Tests showed that the maximum variations in the position of the insects in the plastic cages resulted in differences of about 15 per cent. Normally the difference between a series of counts approximated 10 per cent., because the insects were anaesthetized with carbon dioxide before the cage was placed in the lead castle and were centred on the base of the cage. These wide variations due to the intrinsic failings of the technique preclude the use of the method for exact studies. In the present tests all differences were sufficiently marked to be obvious in spite of these variations. The temperature of the laboratory during the experiments varied from 26 to 30°C.

TABLE 1
 ^{32}P UPTAKE BY GROUPS OF 10 ADULT *OROSIUS*

Duration of Feeding (min.)	Counts per Min. per Insect
1	0
5	0.75
15	1.9
60	17

III. OBSERVATIONS

(a) *Minimum Feeding Time for Detection of the Isotope*

The first experiments were performed to determine the rate of uptake of ^{32}P and the minimum feeding time necessary for the detection of the isotope. This will depend upon the ^{32}P content of the plant, which in this instance approximated 0.1 $\mu\text{c./g.}$ The results are given in Table 1 and demonstrate that measurable amounts could be detected after a period of exposure to a leaf containing ^{32}P of only five minutes. It was apparent from these preliminary results that uptake increased with time, a relationship considered more fully below.

(b) Individual Variation in Amount Ingested

To determine the variability in amount ingested, ten *Orosius* adults were fed together for 30 minutes on a plant containing ^{32}P . The results, determined as described in Section II, are set out in Table 2.

TABLE 2
VARIATION IN AMOUNT INGESTED BY ADULT *OROSIUS*

Sex	F	F	M	M	F	F	F	M	F	M
Counts/min.	17.5	4.5	35.0	9.0	42.0	11.5	11.0	18.5	38.5	35.5

The mean uptake was 22 counts/min., and no difference between the sexes was apparent. The possibility that the differences were due to feeding on different tissues of the plant was disposed of by a series of experiments in which the jassids were fed on solutions of sugars. Table 8 summarizes some data which illustrate this point.

The differences between individuals in food uptake may be due to age, previous feeding, or to other factors that could not be controlled and were not specifically examined. Short periods of starvation (up to 60 min.) prior to the test did not reduce the variability.

(c) Rate of Ingestion

Because of these differences it was clear that an accurate measure of changes in total uptake with time could be obtained by averaging only very large numbers, but the difficulty was overcome by making repeated determinations on the same group. Insects, preferably in groups of five in the feeding cages, can be removed from the leaf on which they are feeding, their radioactivity measured, and they may be replaced on the same leaf. Uptake and excretion are then measured together, and are found to be linear with time over periods of up to three days. This indicates that no injury is caused to the mouth-parts during the removal of the cage from the leaf. Although *Orosius* normally feeds on the phloem (Helson, unpublished data), the rapidity with which they leave a plant when disturbed suggests also that they have no difficulty in removing their mouth-parts from the plant tissues. Counting was generally completed in eight minutes, which represents a small percentage of the total time, so that none of the effects of starvation mentioned below were detectable. Certainly there was generally only insignificant variation from linearity of uptake, which would be expected if the insects fed at a constant rate (Fig. 2). In two comparable experiments, reduced rates of ingestion were observed during certain feeding periods, but during the majority of periods the rate was similar to that reported in Figure 2.

(d) Rate of Excretion

By measuring the cages and insects together as in the previous experiments, and then the insects and cages separately, the amount of ^{32}P excreted may be

determined, since tests made by enclosing living insects in cages between paper "leaves" showed that all excreta produced in the first 30 minutes of feeding was retained in the cages.

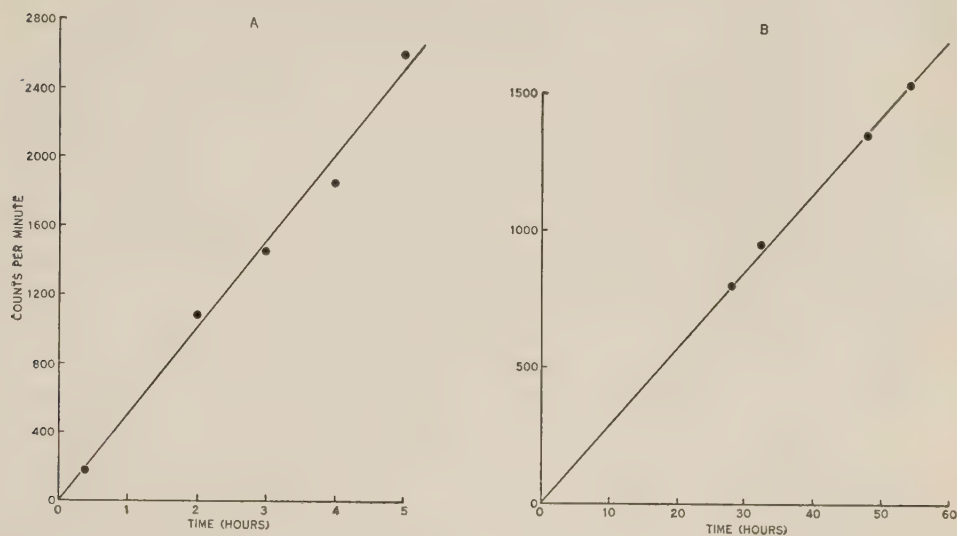


Fig. 2.—Uptake of ^{32}P with time. In Figure 2B the data were corrected for radio-active decay.

Excretion is already abundant 30 minutes after feeding has begun, indicating a rapid rate of passage of materials through the alimentary canal. The ratio of the amount of ^{32}P excreted to amount retained varied considerably between groups (Table 3), but averaged 34.6 per cent. during a 24-hour period.

TABLE 3
AMOUNT OF ^{32}P EXCRETED BY *OROSIUS*

Treatment	Percentage of Ingested ^{32}P Excreted
Fed 30 minutes on radio-active plant	62.5
Fed 30 minutes on radio-active plant	71.7
Fed 24 hours on radio-active plant	41.7
Fed 24 hours on radio-active plant	39.1
Fed 24 hours on radio-active plant	19.1
Fed 24 hours on radio-active plant	32.4
Fed 24 hours on radio-active plant	42.6
Fed 24 hours on radio-active plant	32.9
Fed on radio-active plant overnight— amount excreted during 3 hours' starvation	8.0

It will be observed that the relative amounts excreted during 24 hours are lower than those excreted in 30-minute feeding periods. This observation has not been explained. A further complication arises from the fact that some of

the excreta during the longer period may have been produced by the second method described by Storey and Nichols (1937) and the powdered material so formed was lost. Both methods of excretion have been observed in *Orosius*. The excreted ^{32}P was found to be readily soluble in water.

(e) *Effect of Prior Starvation on Ingestion*

In work on virus transmission it is customary to starve the vectors for short periods before placing them on a plant, because better transmission usually results than if the insects are merely transferred from plant to plant. Jassids, starved in the feeding cages, were able to survive for more than five hours without moisture and for more than 12 hours if kept moist. Starvation for 15-30 minutes increased the ^{32}P uptake but with longer periods the uptake fell off with increasing periods of starvation (Fig. 3).

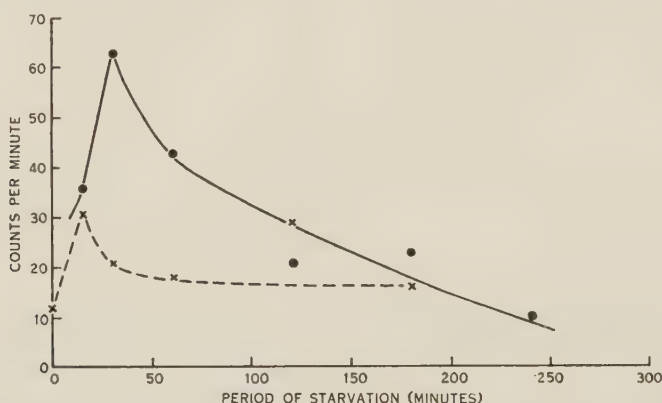


Fig. 3.—Effects of various periods of starvation on quantity ingested by *Orosius* in 30-minute feeding periods.

(f) *Effect of Carbon Dioxide Anaesthesia on Ingestion*

Orosius adults, in common with other small jassids, are not usually easy to handle in such operations as placing them in the feeding cages. But they succumb to and recover from carbon dioxide so readily that their collection and transference are greatly facilitated by using it as an anaesthetic. However, there was a suggestion that such treatment may adversely affect their food uptake. Series of caged adult *Orosius* were therefore set up on the radio-active plants and their total ^{32}P uptake measured at hourly intervals. At various times they were submitted to carbon dioxide anaesthesia before counting. They had recovered by the end of the counting period, and it was found in two experiments that they did not, in fact, ingest measurably less after such treatment (Fig. 4).

(g) *Loss of ^{32}P in Salivary Secretions*

Salivary secretions are generally recognized to be the vehicle in which viruses are transmitted by jassids to plants. It was therefore of interest to determine whether the amount of saliva could be measured either from ^{32}P lost

from the insects or from the isotope injected into the plant. *Orosius* adults were fed on radio-active plants until they contained a high concentration of ^{32}P . They were then transferred to normal beet seedlings and the ^{32}P activity both of the leaves and of the insects determined after various periods of time. No activity could be detected in the plants. Table 4 shows the activity of the insects in two experiments.

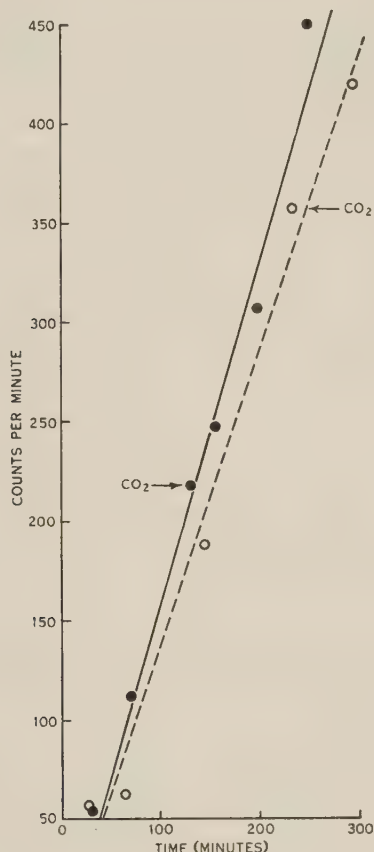


Fig. 4.—Effect of carbon dioxide anaesthesia on subsequent ingestion by *Orosius*. CO_2 administered immediately after measurements indicated by arrows.

It will be observed that the loss in activity approximated the ^{32}P decay curve fairly closely and gives no indication of loss of ^{32}P in the salivary secretions. If such a loss occurs, the amount is within the limit of accuracy of the method.

It was considered possible that, after short periods of feeding, the isotope may be more readily excreted through the salivary glands, and so groups of adult *Orosius* were fed on a radio-active plant for 30 minutes and then immediately transferred to other plants similar except that they contained no ^{32}P . No loss of ^{32}P from the insects could be detected.

Further attempts were made to determine transference of ^{32}P in saliva by inducing the jassids to feed on sugar solutions containing the isotope. Two groups of five jassids were fed overnight on the solutions. The living insects were found to be highly radio-active, and they were then transferred to fresh sugar solutions containing no isotope. After 24 hours' exposure the liquid was

TABLE 4
ACTIVITY OF ^{32}P IN *OROSIUS* FEEDING ON NON-ACTIVE PLANT COMPARED WITH
DECAY OF ACTIVITY OF ISOTOPE

Time	Percentage Activity Remaining	Counts per Min.	
		Group 1	Group 2
0 hours	100	738	344
72 hours	86.5	688	307

withdrawn by a hypodermic syringe. No measurable radio-activity was found in this sugar solution after evaporation. These experiments indicate that the amount of ^{32}P lost in the saliva during feeding is very small.

(h) Absolute Quantity Ingested

Knowing the ^{32}P content and weight of the plant material and the ^{32}P content of the *Orosius*, the weight of material ingested per unit time could be calculated. The plant material was ashed by the method of Piper (1942) and the ^{32}P content of the ashed *Orosius* was measured under comparable conditions. Table 5 gives the results obtained.

TABLE 5
WEIGHT OF PLANT MATERIAL INGESTED BY *OROSIUS* ADULTS

Wt. of Leaf (mg.) (a)	^{32}P Content (counts/min.) (b)	No. of Insects (c)	Period of Exposure to Plant (min.) (d)	^{32}P Ingested (counts/min.) (e)	Calculated Wt. of Material Ingested
					(a) \times (e) (b) \times (c) \times (d)
900	6.0×10^5	6	30	45	0.00037
900	1.0×10^6	5	30	60	0.00036
900	6.5×10^5	5	30	43	0.00040

The mean weight of material ingested is thus calculated to be 0.00038 mg. per min. per insect, or about 2.5 per cent. of the weight of the insect per hour. This figure is too low because not all insects fed all the time. Also the figure may be altered by unequal distribution of the ^{32}P in the leaf.

Comparable experiments in which the feeding time was accurately measured were therefore performed with single insects feeding on sugar solutions. These results (Table 6) indicated that about 0.0024 mg. or about 6.5 times the above figure, were ingested per minute per insect. Observations indicated that the amount of ^{32}P lost by excretion in this series of tests was small.

(i) *Effects of Environmental Conditions on Amount Ingested*

Early in the experiments with artificial diets, attempts were made to determine the effects of temperature, humidity, and light on the amount ingested. Groups of insects were permitted to feed on sugar solutions containing ^{32}P in the light and in the dark at 27°C . and 80 per cent. relative humidity, 25°C . and about 35 per cent. relative humidity, and in the laboratory under higher light intensities. The results were very variable, but considerable amounts were ingested under all conditions tested. However, the lower humidities appeared to result in somewhat higher activities. As was expected from the results presented in Figure 2B, the insects fed readily in the dark.

TABLE 6
WEIGHT OF 1.6 PER CENT. SUCROSE SOLUTION INGESTED BY *OROSIUS* ADULTS

Total Weight of Fluid (mg.) (a)	^{32}P Content (counts/min.) (b)	Duration of Feeding (min.) (c)	^{32}P Ingested (counts/min.) (d)	Calculated Wt. of Material Ingested (mg./min./insect) $\frac{(a) \times (d)}{(b) \times (c)}$
1,800	8.1×10^6	18	435	0.0054
1,400	7.6×10^6	10	114	0.0020
1,500	1.1×10^7	16	234	0.0020
1,200	7.9×10^6	16	207	0.0020
1,400	7.9×10^6	30	119	0.0007

(j) *Effects of Concentration of Sugars on Amount Ingested*

Carter's (1928) mixture of sugars was made up in concentrations from four times to $1/4$ the concentration recommended. Groups of five *Orosius* were exposed on three occasions to these solutions and the activity in the five insects then determined. The results are summarized in Table 7. It will be noted that the results are very variable. The sugar concentration in Carter's solution was optimal in each of the three experiments.

TABLE 7
EFFECT OF CONCENTRATION OF SUGARS ON QUANTITY INGESTED—NUMBERS REFER TO COUNTS PER MINUTE OF FIVE INSECTS

Concentration	Experiment		
	1	2	3
4x	824	206	1380
2x	676	182	546
1x (Carter's solution)	964	430	11834
$\frac{1}{2}\text{x}$	404	140	1146
$\frac{1}{4}\text{x}$	460	62	1752
Distilled water	360	304	382

(k) Effect of Hydrogen Ion Concentration on Amount Ingested

A 1.6 per cent. sucrose solution was made up in distilled water. It had a pH of 5.9. To aliquots, 0.02N NaOH was added drop by drop to give solutions of pH 7.2, 7.8, and 9.0. To similar quantities, 0.02N HCl was added to give solutions of pH 4.2. Tracer quantities of radiophosphorus were added to these and groups of five jassids exposed to the solutions for 24 hours. The radio-activity of each jassid was then determined. The results are presented in Table 8.

The results are extremely variable and do not indicate that the pH of the solution is an important factor in determining the quantity of liquid ingested.

IV. DISCUSSION

The generally accepted hypothesis of the transmission of plant viruses by jassids is due mainly to the work of Storey (1939) and Bennett and Wallace (1938). There is evidence that the virus is ingested, that it passes into the midgut, is absorbed into the blood and makes its way to the salivary glands, in the secretions of which it is injected back into a plant. Ingested phosphorus would not follow the same pathways. It is generally distributed through the body tissues and is readily excreted. Nevertheless, the results presented above

TABLE 8
EFFECT OF pH ON AMOUNT OF SUGAR SOLUTION INGESTED

pH	Insect No.				
	1	2	3	4	5
	Counts per Minute				
4.2	3320	1600	608	444	952
5.9	800	732	620	580	1732
7.2	848	860	476	780	7558
7.8	276	28	636	32	7092
9.0	340	1060	408	308	6955

have some implications for the mechanism of virus transmission. Both isotope and virus can be detected in the insect after five minutes feeding (Grylls, unpublished data); and increasing feeding time or prior starvation results in improved transmission and intensified ingestion. The phosphate ion is mobile and the excretion of it via the saliva would be expected. Transference of isotopic indicators through the saliva of Hemiptera was, in fact, indicated by the observations of Hamilton (1935) and of Carter (1945). In preliminary experiments the green vegetable bug, *Nezara viridula* L., was found to re-inoculate previously ingested ^{32}P into a non-radio-active bean seedling. No explanation of the results with *Orosius* can be suggested, therefore, except that the amount of phosphorus in *Orosius* saliva is too small to be readily detected by the methods used.

Although the variation in ^{32}P uptake between individuals of *Orosius* was considerable, the differences were generally insufficient to account for the disparities observed in the ability of this species to transmit virus. Such differ-

ences may be due, as Storey (1939), Bawden (1950), and others have suggested, to the inability of the virus to penetrate the midgut or to the inactivation of the virus in the insect.

The quantity of material ingested by *Orosius* is small. It indicates that, without the toxic effects that have been demonstrated from the salivary secretions of certain jassids (Carter 1939), or the transmission of phytopathogenic viruses, the feeding of large numbers of jassids would probably not result in considerable damage to plant tissues. Thus, on the basis of the data presented in Table 6, 100 jassids feeding continuously for 24 hours would remove only 0.4 g. of plant material—not a large amount in relation to the transpiration stream.

Fife and Frampton (1936) found that *Eutettix tenellus*, when presented with a choice, fed more readily on alkaline than on acid media. In the above experiments no choice was possible, but the results do not indicate that an acid reaction of the medium renders it unpalatable. Further work will, however, be necessary to determine whether *Orosius* finds the phloem on which it feeds by a pH gradient such as suggested for *Eutettix* (Fife and Frampton 1936).

V. ACKNOWLEDGMENTS

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VI. REFERENCES

- BAWDEN, F. C. (1950).—"Plant Viruses and Virus Diseases." 3rd Ed. (Chronica Botanica Co.: Waltham, Mass.)
- BENNETT, C. W., and WALLACE, H. E. (1938).—Relation of the curly top virus to the vector *Eutettix tenellus*. *J. Agric. Res.* **56** (1): 31-51.
- CARTER, W. (1928).—Transmission of the virus of curly-top of sugar beets through different solutions. *Phytopathology* **18**: 675-9.
- CARTER, W. (1939).—Injuries to plants caused by insect toxins. *Bot. Rev.* **5**: 273-326.
- CARTER, W. (1945).—Oral secretions of the pineapple mealybug. *J. Econ. Ent.* **38**: 335-8.
- FIFE, J. M., and FRAMPTON, V. L. (1936).—The pH gradient extending from the phloem into the parenchyma of the sugar beet and its relation to the feeding behaviour of *Eutettix tenellus*. *J. Agric. Res.* **53**: 581-93.
- HAMILTON, M. H. (1935).—Further experiments on the artificial feeding of *Myzus persicae* (Sulz.). *Ann. Appl. Biol.* **22**: 243-58.
- HELSON, G. A. H.—The transmission of witches' broom virus disease of lucerne by the common brown leafhopper, *Orosius argentatus* (Evans). *Aust. J. Sci. Res. B* **4** (2): 115.
- HILL, A. V. (1941).—Yellow dwarf of tobacco in Australia. II. Transmission by the Jassid *Thamnotettix argentata* (Evans). *J. Coun. Sci. Industr. Res. Aust.* **14** (3): 181-6.
- PIPER, C. S. (1942).—"Soil and Plant Analysis." (Hassell Press: Adelaide.)
- STOREY, H. H. (1933).—Investigations of the mechanism of the transmission of plant viruses by insects. *Proc. Roy. Soc. B* **113**: 463-85.
- STOREY, H. H. (1939).—Transmission of plant viruses by insects. *Bot. Rev.* **5**: 240-72.
- STOREY, H. H., and NICHOLS, R. F. W. (1937).—Defaecation by a jassid species. *Proc. R. Ent. Soc. Lond.* **12**: 149-50.

THE MECHANISM OF SECRETION BY THE SALIVARY GLAND OF THE COCKROACH *PERIPLANETA AMERICANA* (L.)

By M. F. DAY*

[Manuscript received November 13, 1950]

Summary

The two main cell types of the cockroach salivary gland, the ductule-containing cells and the zymogenic cells, are together responsible for the secretion of a powerful amylase and a mucoid substance. The evidence presented suggests that the precursors of both these materials are elaborated in the zymogenic cells and are passed to the ductule-containing cells for excretion. The morphology of the secretory ducts suggests that they also play a part in the elaboration of the saliva.

I. INTRODUCTION

The salivary glands of insects are of special importance in the transmission of protozoal, bacterial, and viral diseases of animals and plants. However, their physiology has been little studied, and, although many functions have been attributed to their secretions, there is comparatively little evidence upon which to base conclusions. As a preliminary to the investigation of more complex salivary glands, those of *Periplaneta americana* (L.) have been examined. Lebedeff (1899) studied these organs in some detail and collated previous data on them, but almost nothing new on the histology of the glands has since been added. He described two types of cells in the glands and concluded that one type produced mucin whereas the other produced the digestive enzyme, and this opinion has never been questioned. Later, Wigglesworth (1927) characterized the enzyme as amylase and found the saliva extremely active in the hydrolysis of starch. He reported invertase to be absent, although it was found in the salivary glands of the cockroach, *Blattella germanica* (L.). Using histochemical methods, Day (1949*b*) confirmed Lebedeff's statement concerning the presence of a mucoid substance in the glands and suggested that the amylase and the mucoid materials were secreted together.

In a consideration of the function of the salivary gland it is necessary to ascribe the secretory products to the correct cell type. But the problem is not as simple as indicated by Lebedeff's report, and an investigation of it is the subject of this paper.

II. MORPHOLOGY OF THE SALIVARY GLANDS

The morphology of the glands, their ducts, and reservoirs of the related *Blatta orientalis* was well described by Miall and Denny (1886), and that of *P. americana* less accurately by Bordas (1897, see his Plate 4, Fig. 3). The

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principal features are illustrated in Figure 1. The acini show an extraordinary superficial resemblance to the salivary glands of vertebrates. The clusters of secretory acini surround the oesophagus and occur at the ends of branching ducts which join with the ducts of the reservoirs and discharge into the salivarium at the base of the hypopharynx. No muscles surround the ducts except the sphincter at the base of the hypopharynx. Nor do the reservoirs have in-

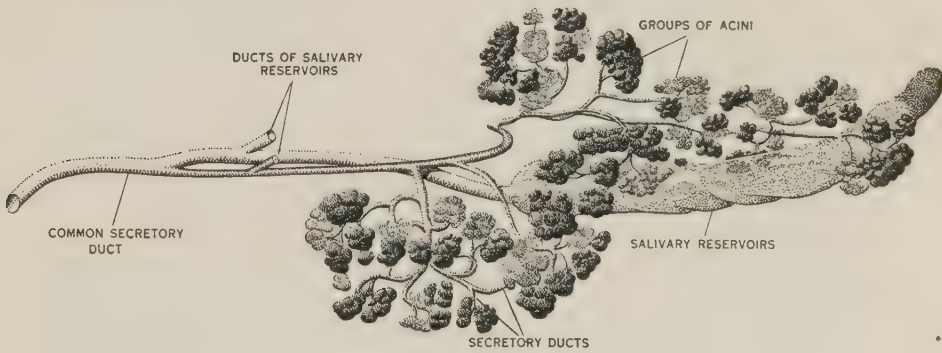


Fig. 1.—Principal features of salivary glands.

trinsic musculature, and so they must be filled by back pressure of those secretory products which are not discharged into the buccal cavity. Similarly, they must be emptied by the pressure of the haemolymph upon their elastic walls. The histology of these is peculiar (Plate 2, Figs. 1 and 2). The ability to expand is provided by the greatly folded epithelium. The wall of the reservoir appears to be composed of the two closely appressed but distinct layers of cells, the outer layer having vacuolated cytoplasm and the inner layer having a prominent chitinous lining. In the dilated reservoir these two layers become almost indistinguishable. This interpretation of the histology of the epithelium of the reservoirs would seem unquestionable, except for one disturbing observation. In the epithelium of the contracted reservoirs, nuclei appear to migrate from one layer to the other and, in fact, some may be constricted in the middle and appear to be partly in both layers. The significance of this is not clear.

III. HISTOLOGY OF THE GLANDS AND THEIR DUCTS

The excretory ducts of the salivary glands and those of the reservoirs both have a thin epithelium, and a taenidia-like lining (Plate 2, Fig. 3). The ducts of the reservoirs retain this structure throughout their length. A few small cells of a fat-body-like tissue surround them over part of their length. The ducts of the glands soon become thick-walled and remain so to the acini themselves (Plate 2, Fig. 4). The cytoplasm of the larger epithelial cells is dense and packed with mitochondria. Characteristic striations occur in the cytoplasm towards the lumen border where the taenidia-like thickening is still distinct, though less marked than in the excretory duct. The nuclei of the epithelial

cells are centrally located. Occasional peripheral nuclei belong to cells of the tracheae which penetrate between the epithelial cells. Intracellular tracheae and tracheoles are very abundant.

Between the acini may be found groups of cells which give every indication of being groups of haemocytes. However, they are of such compact structure that they may be mistaken for definitive organs. A similar structure in another species of cockroach is illustrated by Day (1950, Plate 4, Fig. 24). A study of orcein-stained whole mounts of salivary glands suggests that the acini are surrounded by a connective tissue sheath. In addition, the acini are closely bound together by tracheoles, their extensive innervation and their duct system. An attempt was made to check the shapes of the constituent cells in the glands by dissociation either in Goodrich's (1942) solution, or in hyaluronidase prepared from bull testis by the method of Madinaveitia (1941). The acini are bound together so completely that both these fluids are ineffective.

The acini of the glands are composed of cells of three main types (Plate 1, Fig. 1). The first are the small, centrally located cells of the intercalated ducts. The other two cell types are larger. One type contains striking intracellular ductules. These are Lebedeff's (1899) "peripheral cells" and are homologous with the "parietal cells" of the grasshopper described by Beams and King (1932). The ductules (especially of *Blattella*, but also of *Periplaneta*) are clearly differentiated by soaking the gland in 1 per cent. silver nitrate overnight in the dark, then teasing in glycerine and exposing to sunlight. The ductules always occur in pairs (Plate 1, Fig. 1) and connect with multicellular intercalated ducts in the acinus and eventually with the secretory ducts. The cytoplasm of the ductule-containing cells is finely granular, basophilic, and relatively constant in appearance. The ductules consist of a swollen terminal portion with a striated lining and a narrow neck portion directed centripetally.

The third cell type is the "mucous cell" of Lebedeff or preferably the "zymogenic cell" (Beams and King 1935). The nuclei of these cells are larger than those of the ductule-containing cells and easily distinguished from the other nuclei in the gland. These cells undergo striking changes during secretion, well shown in Bouin-fixed sections stained in Mallory's triple stain. In the non-secreting phase (*a*), which is most marked in individuals in which the glands have been denervated but is also found in starved specimens, the cytoplasm is sparse, acidophilic, and finely vacuolated (Plate 1, Fig. 2A). It gives a weak Bismarck brown stain for mucoids. In the secreting phase (*b*) the cytoplasm first becomes granular, intensely basophilic and gives a strong stain with Bismarck brown (Plate 1, Fig. 2B). Later the basophily is again replaced by material staining with orange G in Mallory's stain and which is apparently discharged as the secretory product (phase *c*) (Plate 1, Fig. 2 C and D). In the final depleted phase (*d*), most of this acidophilic material is discharged and the basophilic substance is produced again. No regenerative cells are present in the acini, so it is clear that complete destruction of cells in the course of secretion does not occur. It will be noted that secretion is asynchronous.

SALIVARY GLAND OF THE COCKROACH

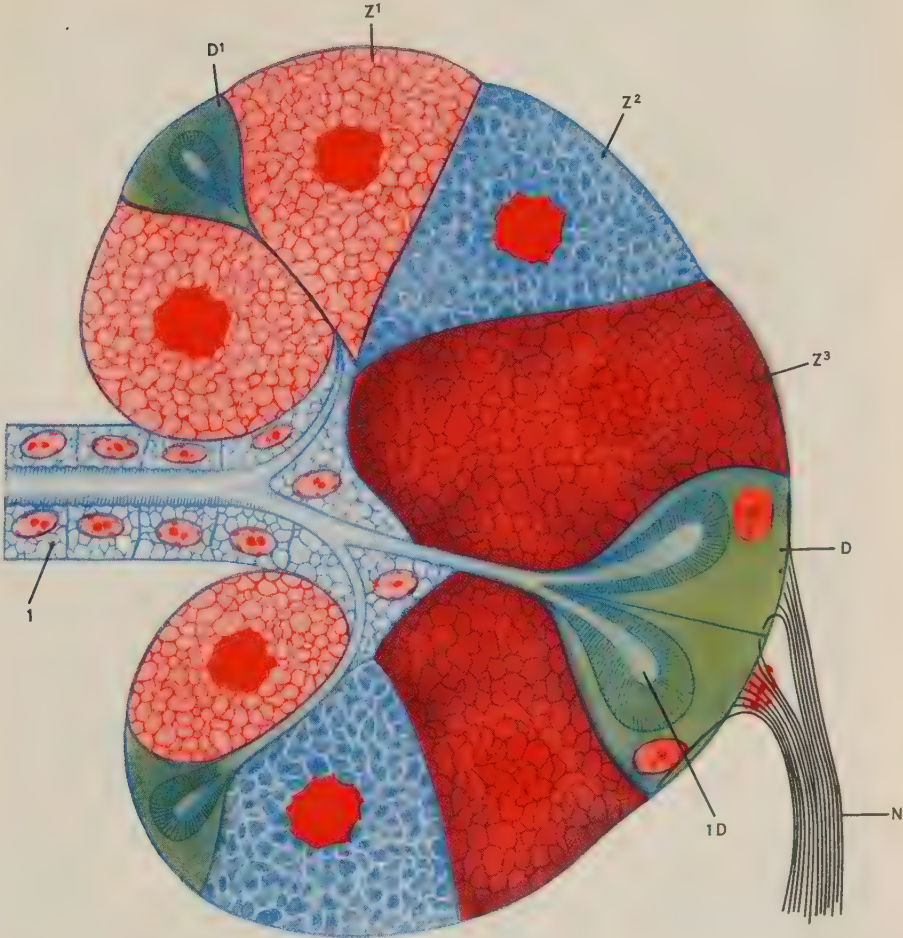


Fig. 1

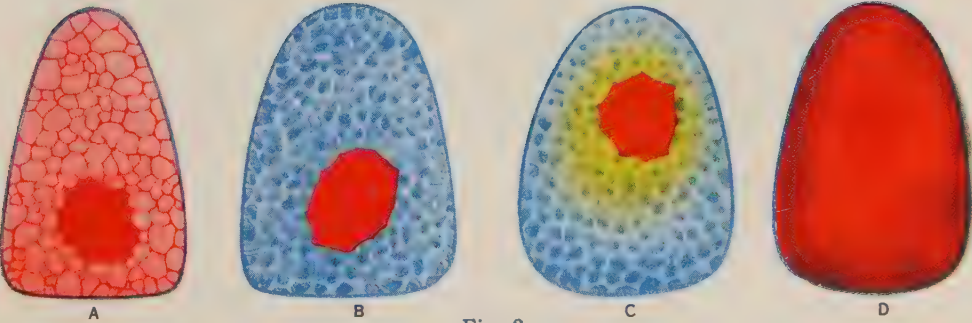
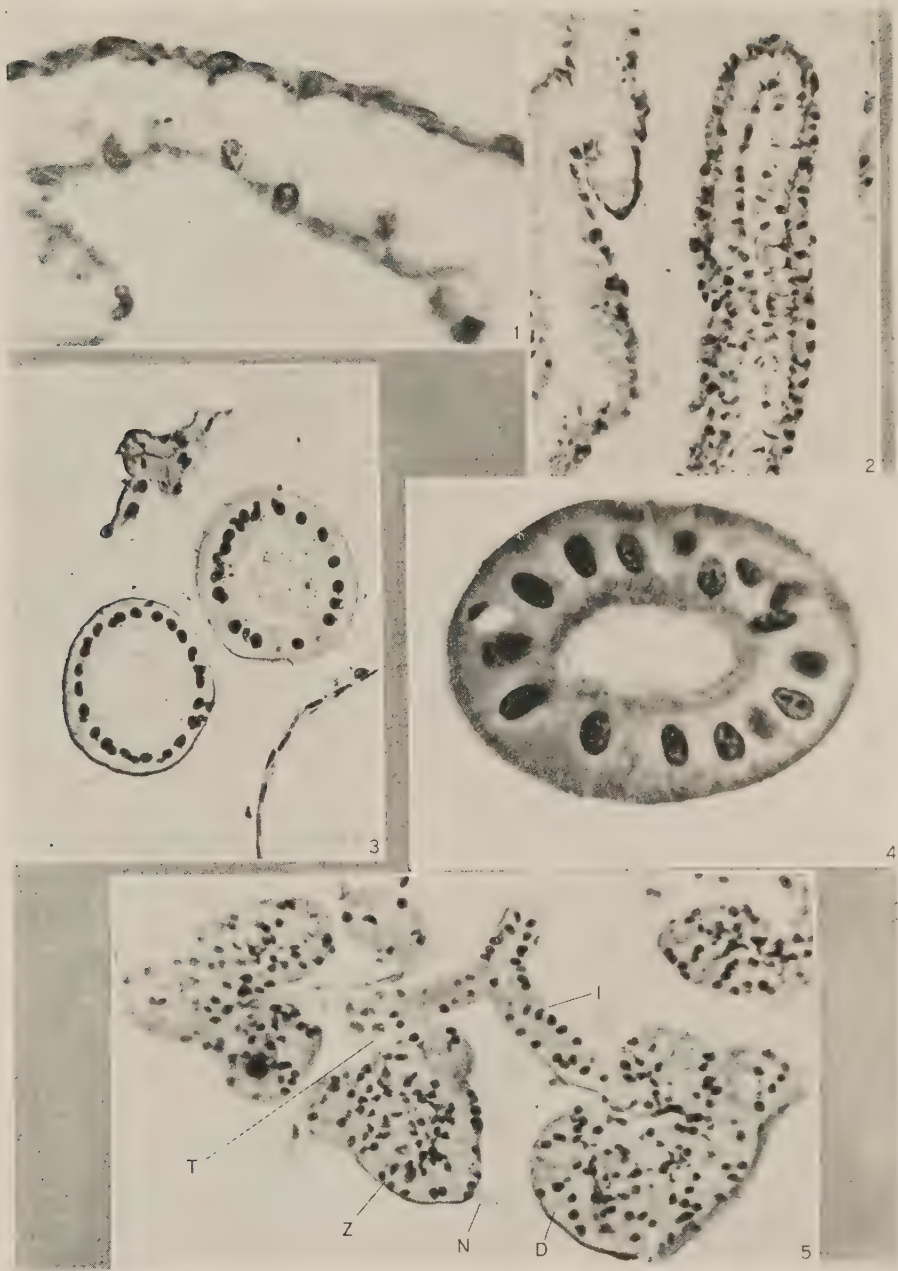


Fig. 2

SALIVARY GLAND OF THE COCKROACH



The histology of the salivary glands of adult *Periplaneta* submitted to a variety of experimental conditions has been studied. Following almost all treatments, some cells can be found in each of the four phases of secretion, but the proportion of cells in each phase varies markedly. The gland of insects starved ten days is mainly in the non-secreting stage. Upon feeding, phases *b*, *c*, and *d* become abundant. Injection of 0.1 ml. of 1:1000 pilocarpine into a *Periplaneta* adult produces characteristic tremors, hesitant gait, and continuous movements of the mouth-parts (cf. Roeder 1939). It produces both cytological evidence of secretion and reduction in the amylase content of the gland. Neither histamine (0.1 ml. of 1:1000 for 30 minutes) nor electrical stimulation from an inductorium of the *nervus recurrens posterior*, the nerve supplying each gland (Nesbitt 1941), produced comparable changes.

An attempt was made to watch the progress of secretory changes by placing dissected glands, either with their innervation intact or severed, in a 1:1000 pilocarpine in Ringer solution under the high powers of the microscope. No changes in the cells were observed either by normal transmitted light or under phase contrast.

The innervation of the acini as seen in methylene blue preparations is very thorough (Day and Powning 1949). Intercellular nerves are well demonstrated by the Boeke technique, and nerve endings are found abundantly on the ductule-containing cells (Plate 1, Fig. 1). If each *nervus recurrens posterior* is severed the insect lives apparently normally for more than 7 days, but the glands become small and consist almost entirely of cells in the non-secreting phase.

IV. ENZYME PRODUCTION

The cytological observations reported above have been related to the phase of secretion of enzyme by performing amylase determinations on one gland of which the other member of the pair was used for cytological study. Only males were employed in these experiments, and the reservoirs and most of the secretory ducts were discarded; also treatments prior to the enzyme estimation were maintained as constant as possible in an attempt to reduce variability in the results, which, in spite of all precautions, was considerable.

Amylase was determined by the Linderstrom-Lang and Holter technique as described by Day and Powning (1949). Arbitrary enzyme units were selected so that the activity of a single gland, from a normal adult male, in 8 ml. of buffer was equivalent to 100 units.

The amylase content of each of the glands from a series of insects was determined separately. It was found that the content of one gland was identical with that of the other member of the pair within the limits of accuracy of the method (± 5 units). (The cytological structure of each of the glands from one insect was likewise similar, although difficult to measure quantitatively.)

Data from an experiment on the effects of various treatments on amylase content are summarized in Table 1. A cytological estimate of the relative number of cells in all secretory phases shows beyond doubt that there is no

correlation between amylase activity and the number of cells in any secretory phase. High or low enzyme activity may be associated with marked acidophily or basophily of the cytoplasm of the zymogenic cells. However, high enzyme content was always associated with marked cytological activity of the cells in phases ($b + c$), suggesting that the zymogenic cells were responsible for the production of amylase. These conclusions were checked with a larger series of 75 insects of both sexes submitted to a variety of treatments (pilocarpine, histamine, starvation, denervation, etc.).

TABLE 1
AMYLASE CONTENT AND CYTOLOGY OF SALIVARY GLANDS OF ADULT MALE *PERIPLANETA*

Treatment	Amylase Units	Cytological Appearance No. of Cells out of 10 in:		
		Phase <i>a</i>	Phase <i>b</i>	Phase (<i>c</i> + <i>d</i>)
Starved 7 days	37	3	1	6
	50	4	5	1
	46	4	5	1
	50	4	3	3
Fed only water 7 days	36	4	2	4
	20	4	1	5
	40	3	2	5
Fed only starch 7 days	100	1	5	4
	32	3	1	6
	37	4	1	5
	140	4	1	5
Fed normal diet	112	2	3	5
	180	3	1	6
	120	3	3	4
	95	6	1	3

In both of these series, some slides were stained with Bismarck brown and some with Mallory's triple stain. Comparison of these shows clearly that the basophilic substance of phase *b* and the acidophilic substance characteristic of phase *c* both give the test for mucoid substances. Further confirmation of the nature of these materials was obtained by incubating acetone-fixed slides in hyaluronidase (Dempsey *et al.* 1947). Some slides so treated were used as a source of enzyme for the digestion of starch solutions. There was considerable loss in activity due to the treatment but sufficient remained to give a marked difference between the amount of amylase extracted from control slides and from similar slides incubated for 30 minutes at 37°C. in a preparation of hyaluronidase. Subsequently both series were stained in Mallory's triple stain. The acidophilic substance, and only this, was noticeably depleted by the treatment with hyaluronidase. The contents of the zymogenic cells give a positive Hotchkiss test for polysaccharides (periodate-Feulgen reaction). The evidence suggests, therefore, that the zymogenic cells produce the mucoid substance.

Thus, of the several lines of evidence presented, none is in disagreement with the conclusion that the mucoid substance and the amylase are both elaborated by the zymogenic cells. If this hypothesis is correct, the function of the ductule-containing cells requires consideration. The distribution of alkaline phosphatase in the glands suggests an explanation. This enzyme was found to be confined in the salivary glands of *Blattella* to those parts of the zymogenic cells surrounding the neck portion of the ductules (Day 1949a). Its distribution in *Periplaneta* is the same. In view of the suggested action of alkaline phosphatase in the transference of materials across cell boundaries, and because the distribution of the enzyme in many insect tissues is explained by such an action, it seems reasonable to assume that such is its function in the acinus. This evidence, together with that on the morphology and innervation of the ductule-containing cells, points to their dominant role in the excretion of the secretory products.

V. THE ROLE OF THE SECRETORY DUCTS IN THE FORMATION OF SALIVA

Neither the saliva from the mouth-parts nor the contents of the reservoirs give the same staining reactions as the contents of the cells of the acinus. It is reasonable to suggest, therefore, that the ducts perform a role in the production of the saliva. The following facts support this contention:

(1) The histology of the ducts, particularly the cytoplasmic striations and the intracellular striations, certainly indicates some function other than a purely mechanical one of transporting the saliva from the acinus to the mouth-parts or reservoir. This is clearly suggested by a comparison between the structure of the ducts of the glands with those of the reservoirs. The latter apparently function only in conduction.

(2) The presence of many mitochondria in the epithelial cells of the ducts suggests that they perform a secretory function.

(3) A non-specific esterase, although completely absent from the cells of the acini, is demonstrable in low concentration in the ducts by the histochemical method of Nachlas and Seligman (1949). Although its function is problematical its presence suggests again some function other than a purely mechanical one.

There is very little information on the composition of the saliva as ejected over the mouth-parts. It is a clear liquid containing an active amylase. On evaporation, conspicuous crystals are deposited. These give a strong positive test for chloride. The saliva is not viscid, nor does it give any histochemical test for mucoid substances.

VI. DISCUSSION

The hypothesis of the secretion of saliva which emerges from the observations and experiments mentioned above is as follows. The secretory products consist of a mucoid substance and a powerful amylase. (The possibilities that they are one and the same or that the mucoid substance is the enzyme precursor are worth considering.) Precursors of both mucoid and enzyme originate

in the zymogenic cells. They, or products elaborated from them, are passed through the cell walls to the ductules and thence through the intercalated ducts to the secretory ducts, where further changes occur, until the completed saliva is either excreted on to the mouth-parts or passed to the reservoir.

This hypothesis does not suggest the mechanism whereby nerves innervating the ductule-containing cells cause them to excrete their contents or replenish them from the zymogenic cells. Nor is the possibility eliminated that the ductule-containing cells contribute some substances to the saliva. The work of Beams and King (1935) on the grasshopper salivary glands suggests that the homologous cells in these insects do, in fact, produce a component of the salivary secretions.

The histochemical demonstration that the zymogenic cells produce the mucoid substance is in agreement with the original suggestion of Lebedeff (1899). However, the assignment of the amylase precursors also to the zymogenic cells is not in agreement with Lebedeff's views and the evidence for this conclusion is therefore summarized, as follows:

(1) The amylase is the most significant component of the saliva and it is reasonable to suggest that it is produced from the dominant cells of the acinus.

(2) The cytological activity of the zymogenic cells is marked when high enzyme concentration is recorded.

(3) The reduction in enzyme following pilocarpine is correlated with changes that were more marked in the zymogenic cells than in the ductule-containing cells.

(4) The reduction of material in the zymogenic cells by the action of hyaluronidase was accompanied by loss of amylolytic activity.

It is appreciated that none of these lines of evidence is conclusive, but together they strongly suggest that the zymogenic cells are the source of amylase of the saliva, and thus confirm the opinion of Hofer (1887).

The action of pilocarpine in causing secretion in *Periplaneta* is noteworthy. This parasympatheticomimetic drug causes salivary secretion in vertebrates and also in a number of insects (Lebedeff 1899; Oka 1930). The superficial resemblance between the salivary glands of vertebrates and some insects mentioned above is thus matched by some, perhaps fortuitous, physiological similarities.

VII. ACKNOWLEDGMENTS

Valuable technical assistance was rendered by Mr. T. D. C. Grace. Thanks are due to Mr. L. A. Marshall for preparing Figure 1 and Plate 1.

VIII. REFERENCES

- BEAMS, H. W., and KING, R. L. (1935).—The architecture of the parietal cells of the salivary glands of the grasshopper with special reference to the intracellular caniculi, Golgi bodies, and mitochondria. *J. Morph.* **53** (2): 223-41.
- BORDAS, L. (1897).—L'appareil digestif des Orthoptères. *Ann. Sci. Nat.* **8** (5): 1-208.

- DAY, M. F. (1949a).—The distribution of alkaline phosphatase in insects. *Aust. J. Sci. Res. B* 2: 31-41.
- DAY, M. F. (1949b).—The occurrence of mucoid substances in insects. *Aust. J. Sci. Res. B* 2: 421-7.
- DAY, M. F. (1950).—The histology of a very large insect, *Macropanesthia rhinocerus* Sauss. (Blattidae). *Aust. J. Sci. Res. B* 3: 61-75.
- DAY, M. F., and POWNING, R. F. (1949).—A study of the processes of digestion in certain insects. *Aust. J. Sci. Res. B* 2: 175-215.
- DEMPSEY, E. W., *et al.* (1947).—The dye-binding capacity and other chemo-histological properties of mammalian mucopolysaccharides. *Anat. Rec.* 98: 417-29.
- GOODRICH, E. S. (1942).—A new method for dissociating cells. *Quart. J. Micr. Sci.* 83: 245-58.
- HOFER, B. (1887).—Untersuchungen über den Bau der Speicheldrüsen und des dazu gehörenden Nervenapparates von *Blatta*. *Nova Acta Leop. Carol* 51: 345-95.
- LEBEDEFF, A. (1899).—Über die Speicheldrüsen der Küchenschabe (*Periplaneta orientalis* L.) *Trud. Kazan Univ.* 33: 1-20. (In Russian, 3 pp. German summary.)
- MADINAVEITIA, J. (1941).—Diffusing factors. 7. Concentration of the mucinase from testicular extracts and from *Crotalus atrox* venom. *Biochem. J.* 35: 447-52.
- MIALI, L. C., and DENNY, A. (1886).—"The Structure and Life History of the Cockroach (*Periplaneta orientalis*)."
(Lovell Reeve: London.)
- NACHLAS, M. M., and SELIGMAN, A. M. (1949).—The histochemical demonstration of esterase. *J. Nat. Cancer Inst.* 9: 415-25.
- NESBITT, H. H. J. (1941).—A comparative morphological study of the nervous system of the Orthoptera and related orders. *Ann. Ent. Soc. Amer.* 34: 51-81.
- OKA, H. (1930).—Untersuchungen über die Speicheldrüse der Libellen. *Z. Morph. Okol. Tiere* 17: 275-301.
- ROEDER, K. D. (1939).—The action of certain drugs on the insect central nervous system. *Biol. Bull.* 76 (2): 183-89.
- WIGGLESWORTH, V. B. (1927).—Digestion in the cockroach. *Biochem. J.* 21: 791-811.

EXPLANATION OF PLATES 1 AND 2

PLATE 1

- Fig. 1.—Semidiagrammatic section of acinus of salivary gland of *Periplaneta*. *I*, intercalated duct; *Z¹-Z³*, zymogenic cells in phases of secretion; *D*, ductule-containing cell; *ID*, Intracellular ductule; *N*, nerve.
- Fig. 2.—*A-D*, phases in secretion of zymogenic cells; *A*, regenerating or non-secreting phase. Note acidophilic, finely vacuolate cytoplasm; *B*, early secreting phase. Cytoplasm granular and intensely basophilic; *C*, late secreting phase. Basophilia partly replaced by acidophilic cytoplasm; *D*, excreting phase. Cytoplasm completely acidophilic.

PLATE 2

All figures are photomicrographs of the salivary glands and associated organs of *Periplaneta americana* (L.), taken with a Leica photomicrographic attachment.

- Fig. 1.—Section of the dilated salivary reservoir.
- Fig. 2.—The same in the contracted condition.
- Fig. 3.—Section of excretory ducts.
- Fig. 4.—Section showing secretory ducts. Note taenidia-like thickenings, striated peripheral cytoplasm, central nuclei, dense cytoplasm, and intercellular trachea.
- Fig. 5.—Section of acini of gland showing non-secreting resting phase. Note cytoplasm of zymogenic cell (*Z*), ductule-containing cell (*D*), intercalated ducts (*I*), tracheae (*T*), and nerves (*N*).

THE OCCURRENCE OF BARIUM AND STRONTIUM IN INSECTS

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Summary

Barium and strontium may be detected histochemically by the formation of reddish compounds following the treatment of tissues with sodium rhodizonate. Calcium does not appear to react under the neutral conditions employed in the test. Treatment with chromate inhibits the reaction with barium, but not with strontium, providing a means for distinguishing between these elements. Permanent histological sections can be produced in the usual manner, so long as a neutral fixative is employed.

Both barium and strontium were detected in the tissues of many insects. They occurred most frequently in the malpighian tubules, less often in the midgut and reproductive organs, and very occasionally in the hindgut and fat body. It is probable that both elements are absorbed in the midgut of most insects. The distribution of barium and strontium was characteristic for each species. When staining occurred it was almost always of granules, which frequently had a characteristically restricted distribution within the cell. Nucleoli stained occasionally, although the remainder of the nucleus seldom reacted.

The possible importance of barium and strontium in insect metabolism is discussed. However, the granules detected are regarded as being either reserves for future use or as a form of storage excretion.

I. INTRODUCTION

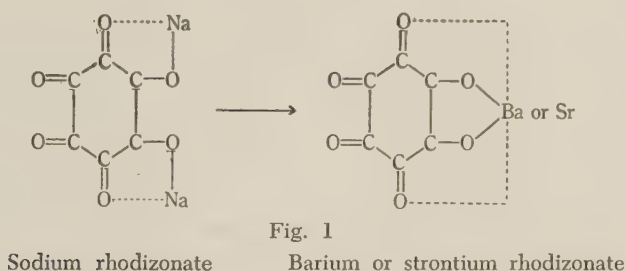
In the course of an investigation into factors influencing the formation, in the malpighian tubules of blowfly larvae, of granules containing calcium and magnesium salts (Waterhouse 1950), it became desirable to determine whether the accumulating mechanism was specific for these metals or whether, in addition to calcium, other members of the alkaline earth series could be stored. Strontium, for example, is considered to follow a path in the body similar to that of calcium (Norris and Kisielecki 1948). Further, it is known that absorption of barium can take place in the blowfly larval midgut (Waterhouse 1945). If present in the diet, barium would thus be available for granule formation. A search was therefore made for suitable histochemical methods for the detection of barium and strontium. The only method available appears to be Cretin's reaction (Lillie 1948), which is said to give a green lake with both barium and strontium, although this reaction was not sensitive enough to give a positive result when applied to blowfly larvae. An extremely sensitive method for the histochemical detection and differentiation of barium and strontium, based on the formation of coloured rhodizonates, was therefore developed. When

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both barium and strontium were demonstrated in certain regions of the malpighian tubules of blowfly larvae raised on a diet not specially enriched with these elements, the test was applied to a number of other insects to determine whether or not accumulation of these metals was of general occurrence.

II. METHODS

The staining method developed for the detection of barium and strontium is based on the reaction between these metals and sodium rhodizonate, with the formation of relatively insoluble red to reddish brown rhodizonates. This reaction was first described by Feigl (1924) and has since been used as a 'spot test' which is reliable under most conditions (B.D.H. 1946; Feigl 1943, 1947). The reaction is considered to be as shown in Figure 1, the intensification of colour being due to the presence of a 5-membered ring in which the metal atom is coordinated by auxiliary valencies to the oxygens of the adjacent C = O groups (Feigl 1943).



Many metals form coloured rhodizonates (Feigl 1943). However, only barium and strontium produce red precipitates, and these are difficult to distinguish from one another under the conditions of test outlined below. Two elements that frequently occur at sites of rhodizonate staining, namely calcium and magnesium, do not appear from available chemical evidence to interfere with the test for barium and strontium. Magnesium does not produce coloured rhodizonates under any conditions and, although calcium reacts under alkaline conditions it does not do so under neutral conditions (Feigl 1943). Neutral conditions are employed in the present method. Calcium hydroxide and oxide produce brownish red precipitates, but many other calcium salts do not, for example the chloride, carbonate, sulphate, oxalate, and several of the many calcium phosphates. This can be readily demonstrated by immersing these salts in a solution of sodium rhodizonate. Whereas some or all of the latter group of calcium compounds occur in the insect body, it is most unlikely that the hydroxide and oxide do so because of their comparatively high alkalinity. It is very important for these tests to use pure calcium salts, free from barium and strontium, or misleading results will be obtained. Thus 'Analar' calcium chloride gives with rhodizonate a distinct red colour but no precipitate, whereas spectroscopically pure calcium chloride* does not react.

* Johnson Matthey & Co. Ltd., London.

There are several differences in the reactivity of barium and strontium salts that can be used to differentiate between these two metals. The most important for the present purpose is that strontium chromate reacts with sodium rhodizonate, whereas barium chromate does not. Other useful features are that strontium carbonate does not react, but barium carbonate does, and that barium rhodizonate reacts with sulphate to form barium sulphate.

In the tests to be described, the gross distribution of barium and strontium was determined by immersion of the tissues for several minutes in 70 per cent. alcohol (to facilitate penetration), followed by a brief wash in distilled water and then immersion in freshly prepared 1 to 2 per cent. solution of sodium rhodizonate* in distilled water or in $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ buffer at pH 7. A saturated solution in dilute alcohol can also be used. With the aqueous staining solutions the colour takes 30 minutes or more to develop to full intensity. After several hours the tissues may commence to disintegrate and the stain sometimes fades. On standing, rhodizonate solutions oxidize and the typical yellow colour fades. The tissues were examined for the presence of red staining after washing out excess sodium rhodizonate with 70 per cent. alcohol. Complete removal sometimes takes several hours and several changes of alcohol.

Permanent mounts were made by dehydrating, clearing, and mounting the stained tissues. For the preparation of sections any fixative is satisfactory that neither dissolves barium or strontium nor forms barium or strontium complexes less soluble than their rhodizonates. Acid fixatives (e.g. Bouin, Carnoy, etc.), which dissolve the reactive granules (see later), are therefore quite unsatisfactory. A good fixative is 10 per cent. neutral formalin in 70 per cent. alcohol, but even this fixative must be tested to ensure that it is neutral or slightly alkaline. If it is slightly acid, as may happen if formalin is "neutralized" by shaking for too short a time with calcium carbonate, the fixed tissues will no longer stain for barium or strontium. Overfixation, even in neutral fixative, is to be avoided, and 1 to 2 hours is usually quite adequate for insect tissues. After fixation and thorough washing in alcohol, it is most convenient to stain the tissues in bulk with sodium rhodizonate before sectioning. There is rather great variability (see later) in the amount of barium and strontium present and this procedure ensures that the tissues selected for sectioning contain sufficient of these metals to provide a visible stain. Staining after sectioning often gives negative results unless the sections are thick. This is because the reacting granules are frequently lost from the sections during the relatively long period of immersion in aqueous rhodizonate. Any of the usual counterstains may be used after sectioning, those producing a blue or green coloration providing most contrast. Immersion in a saturated solution of water-soluble aniline blue in 70 per cent. alcohol provides a convenient counterstain.

The most satisfactory method for differentiating between barium and strontium is to immerse the tissue after rhodizonate treatment in saturated aqueous potassium chromate. If the intensity of staining diminishes, barium is present, barium chromate being less soluble than barium rhodizonate. If

* B.D.H. spot test reagent.

some staining remains after chromate treatment, strontium is present, strontium chromate being more soluble than strontium rhodizonate. Alternatively the chromate treatment may be applied before staining in rhodizonate, or a little chromate may be added to the rhodizonate staining solution. Either procedure will prevent barium from producing a red precipitate.

Tetrahydroxyquinone has reactions similar to those of sodium rhodizonate except that, in addition to strontium, calcium and magnesium are reported to react (Yoe 1938). In order to provide confirmation of rhodizonate staining, the tissues of several insects (*Blattella*, *Apis*, *Culex*) were immersed in a concentrated aqueous solution containing tetrahydroxyquinone and potassium chloride instead of in sodium rhodizonate. The same distribution of staining occurred as with rhodizonate, thereby demonstrating the presence of one or more of the above four reactive metals.

III. RESULTS

(a) *Specificity of the Rhodizonate Reaction*

The results obtained with the blowfly *L. cuprina* will be considered first, because the validity of the method of detection of barium and strontium was principally tested with this species.

When third-instar larvae, reared on liver or on a medium consisting of egg white, yeast, and sodium chloride (Lennox 1939), were treated with rhodizonate solution, intense cherry-red staining generally occurred only in the regions of the malpighian tubules specialized for the accumulation of granules (Fig. 2A). These are the blind distal halves of the two tubules, which discharge via a common duct into the left side of the alimentary canal (Waterhouse 1950). The regions of the tubules not specialized for granule formation, and having a yellow coloration typical of most insect tubules, generally stained comparatively lightly, if at all. The reaction of the granule-accumulating region was due to the presence in the lumen of the tubule of granules varying from pink to dark red. A varying number of granules remained unstained when they were smeared on a slide before rhodizonate treatment, but almost all showed some staining if treated *in situ*.

When granules, smeared on a slide, were treated with Gallamine blue, which forms a specific purple lake with calcium (Stock 1949), all or almost all of the granules became stained, indicating the presence of calcium. This suggests that calcium may be an interfering metal. However, not only are calcium and rhodizonate reputedly unreactive (Feigl 1943), but there is no increase in staining of larvae fed on calcium-enriched media. Such larvae accumulate massive deposits of granules that are far richer in calcium than those formed on ordinary diets (Waterhouse 1950). It appears improbable, therefore, that rhodizonate staining is due to some unrecorded reaction with unusual calcium salts that may be present. Larvae fed on magnesium-enriched media accumulate granules extremely rich in magnesium and relatively poor in calcium and these stain similarly to the calcium-rich granules, although magnesium does not

react with rhodizonate (Feigl 1943). With regard to other metals, no increased staining was observed when larvae were reared on media containing added soluble salts of lead, zinc, cadmium, mercury, tin, or copper. All of these, and particularly lead, are possible interfering metals (Feigl 1943). Ammonium salts also produce a brown rhodizonate *in vitro*, but the distribution of rhodizonate staining in *Lucilia* larvae is quite unlike the distribution of ammonia (Lennox 1941), indicating that ammonia does not interfere under

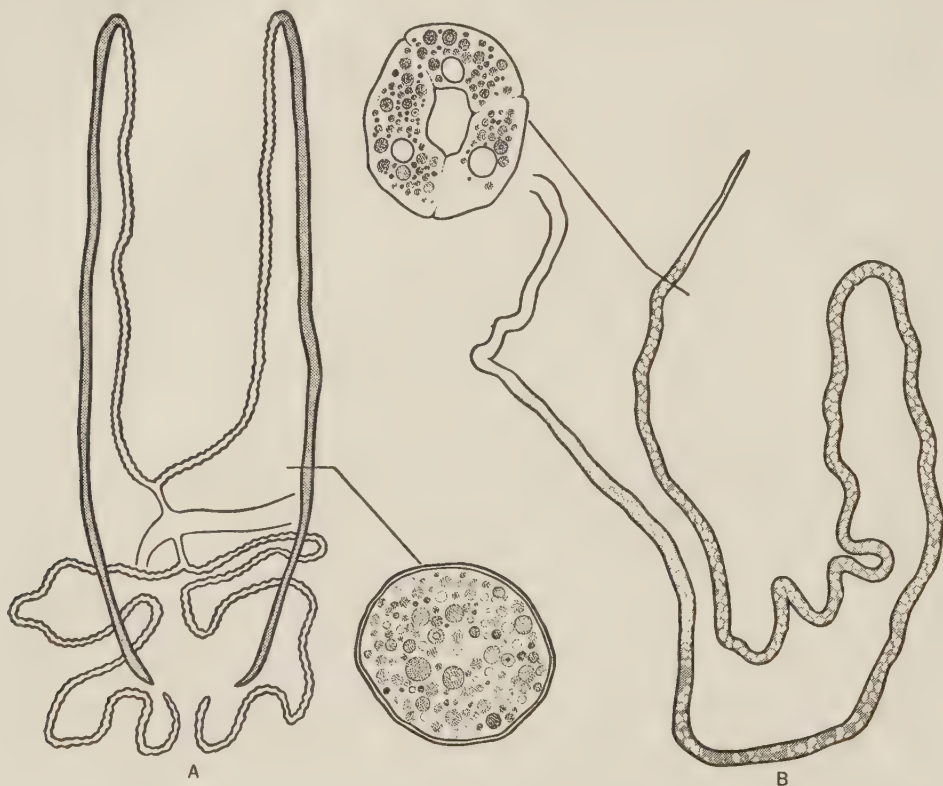


Fig. 2.—Distribution of rhodizonate staining in the malpighian tubules of *Lucilia cuprina* larva, (A), and *Blattella germanica* (B). The presence of barium and strontium is indicated by stippling.

the conditions of test. On the other hand, the addition of barium or strontium to the medium, or the injection of dilute solutions in saline into the haemolymph, very greatly intensifies the staining of the tubules. This increased staining was indistinguishable in character from that of larvae on control medium, which suggests that barium, strontium, or a material possessing identical staining reactions, is present in control larvae. Certainly it is safe to say that, when barium and strontium are available, they are accumulated in the granules.

Very occasionally, on barium- or strontium-enriched media, red staining could be seen in a narrow band of cells in the middle of the midgut (Waterhouse 1945), in the midgut caeca, and in some parts of the fat body, notably

those adjacent to the granule-accumulating regions. The yellow regions of the tubules may stain quite heavily.

Evidence of the presence of both barium and strontium in control larvae was obtained by conversion of both metals into their chromates, followed by treatment with sodium rhodizonate. This procedure prevented the formation of the very intense coloration in the tubules of barium-fed larvae, but did not influence the coloration of larvae fed on strontium-enriched medium. When control larvae were pre-treated with chromate, subsequent staining of the tubules with rhodizonate was considerably less intense than in untreated larvae, indicating the presence of both barium and strontium.

As an independent check on the validity of the staining reactions, the granule-accumulating regions of the tubules from 500 *L. cuprina* larvae were analysed spectrographically. Barium was found to be present in a concentration between 0.001 and 0.005 per cent. dry weight, and strontium between 0.01 and 0.02 per cent. dry weight. Since the rhodizonate reaction is considerably more sensitive for barium than for strontium (Feigl 1947), the analyses are in no way inconsistent with the relative staining intensities ascribed above to these two elements. It is interesting to note that the level of strontium in the granules is of the same order as that recorded for bone ash (Hodges *et al.* 1950).

TABLE 1
SPECTROGRAPHIC EXAMINATION OF INSECT TISSUES FOR BARIUM AND STRONTIUM

Insect	Portion of Insect	Ba	Sr
<i>Apis mellifica</i> (worker)	Midgut	Present	Present
	Hindgut and malpighian tubules	Trace	Trace
<i>Periplaneta americana</i> (adult)	Midgut and hindgut	Trace	Present
	Malpighian tubules	Trace	Present
<i>Pieris rapae</i> (adult)	Midgut	Not detected	Not detected
	Malpighian tubules	Present	Present
	Hindgut and reproductive organs	Present	Present

A qualitative spectrographic examination was also carried out of 1.5N HCl extracts of tissues from three other insects (Table 1). Both barium and strontium (which is more easily detected spectrographically) are present in the honey bee, the American cockroach, and the cabbage white butterfly. Comparison of these results with those for rhodizonate staining (Table 2) indicate a close agreement. There are two exceptions, namely that strontium was detected in the midgut and hindgut of *Periplaneta*, which seldom stain, and that neither barium nor strontium was detected in the *Pieris* midgut, which often stains, although seldom intensely. Possible explanations for the former are that some strontium compounds may not react, or that the strontium is diffuse

and not present in granule form (see later). It is highly probable that, in the *Pieris* midgut, the weight of sample obtained was too small for the detection of barium and strontium by spectrographic means. Experiments with radio-active barium and strontium to be discussed later indicate that both metals are distributed in the insect body in exactly the fashion demonstrated by the rhodizonate staining reaction. There appears to be good evidence, therefore, that the staining observed is due to the presence of barium and strontium rhodizonates and not to other metal rhodizonates.

(b) *Distribution of Barium and Strontium in Representative Insects*

A number of insects, fed on their normal diets, were examined. As can be seen in Table 2, both barium and strontium are present in histochemically detectable amounts in representatives of most orders, barium being demonstrated more frequently than strontium. There is little doubt, therefore, that these elements are normal trace constituents of most insects.

Red staining occurred most frequently in the malpighian tubules, less frequently in the alimentary canal and reproductive organs, and very occasionally only in the fat body. Other organs and tissues did not stain. Transference of insects from their normal diet to one free of barium and strontium, such as 5 per cent. A.R. sucrose solution, did not result in a rapid reduction in staining intensity, indicating that the accumulations are fairly static in nature.

Malpighian tubules.—The distribution of barium and strontium in the malpighian tubules is often characteristically confined to a particular zone. We have noted earlier in *Lucilia* larvae, for example, that staining occurs principally in the blind granule-accumulating portions of one pair of tubules (Fig. 2A). A similar restricted staining occurs in many other Diptera. In Orthoptera, Isoptera, and Dermaptera there is a second type of distribution. The short blind tip region of each tubule does not stain. This region changes abruptly to one of fairly uniform, intensely stained cells (forming about 4/5 of the tubule), and this is followed in turn by a weakly staining or unstained portion discharging into the gut (Fig. 2B). Some tubules show little or no staining. Lison (1942) observed in *Periplaneta* and *Forficula* that, following the injection of certain dyes into the haemolymph, they were discharged into the lumen of the malpighian tubules; later they were reabsorbed from the lumen by the tubule epithelium and appeared as granules in the cells of a particular region of the tubule. In *Periplaneta* this region coincided with that which stains with rhodizonate. The agreement was also good between *Forficula* and the two species of Dermaptera examined in the present study. It is possible that the same mechanism is responsible for the 'athrocytosis' of the dyes and for the formation of the granules containing barium and strontium.

A third type of distribution was encountered in some insects (e.g. *Heteronympha*) in which all of the tubule except the region leading into the gut was frequently stained. In most insects the tubules associated with the lower portion of the hindgut stained more intensely than those serving other regions of the body.

No barium or strontium could be detected in the tubules of some insects, e.g. *Ctenolepisma* and *Tenebrio*, although barium could often be detected in the midgut of *Ctenolepisma* and was quite intense in the midgut of adult *Tenebrio* after feeding on a barium-enriched food. Staining occurred only very rarely in the tubules of *Apis* although both barium and strontium were abundant in the midgut. It is interesting to note that mature *Nasutitermes* workers invariably had barium and strontium in their tubules whereas the immature workers did not stain at all. Staining occurred in only about 30 per cent. of mature soldiers, and in a rather higher proportion of penultimate instar soldiers. No staining occurred in a king or a queen, although it did in immature reproductives with well-developed wing buds. These differences may be a reflection of different feeding habits. The mature workers attack wood, whereas the other castes and the immature workers are fed by the mature workers on faecal material and on a regurgitate from the alimentary canal. It thus appears that some materials may already have been removed even from the latter food, unless of course the physiology of excretion is very different in different castes.

When barium and strontium are present in the tubules, they generally occur as constituents of the granules that are frequently so abundant in the cytoplasm of the epithelial cells. These granules are soluble in many of the common histological fixatives and, therefore, seldom appear in sections. They are, however, readily seen in living preparations. The granules that stain with rhodizonate are frequently distributed fairly uniformly throughout the central zone of the cytoplasm (e.g. *Nasutitermes* (Plate 2, Fig. 5), *Blattella* (Plate 2, Fig. 4)). They seldom occur immediately adjacent to the lumen and very seldom adjacent to the haemocoel borders of the cell. There is no evidence, therefore, that these cytoplasmic granules are ever excreted. However, in all the Diptera examined and in some adult Lepidoptera (*Pieris*, *Heteronympha*), stained granules occur in the tubule lumen and these are, doubtless, eliminated. Tubule nuclei generally remain unstained, although occasionally light staining has been observed in some species (*Blattella*, *Pieris*, *Heteronympha*, *Metiorhynchus*).

Alimentary canal

Foregut.—No staining could be demonstrated in the foregut epithelium of any of the insects studied.

Midgut.—In a number of insects the midgut epithelium became stained. There was often a considerable variation in the staining intensity between individuals of a single species. This is presumably due to slight differences in the composition of the food ingested.

The distribution of staining followed no uniform pattern. In some insects (e.g. *Apis*, *Chrysopa*, adult *Tineola*) the midgut stained fairly uniformly throughout; in others (e.g. *Tenebrio*) no staining was observed at all; in others again only portion of the midgut stained. Thus in *Ctenolepisma* only the caeca and the anterior half of the midgut stained; in *Nezara* only the first of the five regions of the midgut ever reacted; and in *Periplaneta* only the caeca stained

TABLE 2
GROSS DISTRIBUTION OF HISTOCHEMICALLY DETECTABLE BARIUM AND STRONTIUM IN INSECT TISSUES

Insect	Malpighian Tubules	Midgut	Hindgut	Fat Body	Reproductive System
Thysanura					
<i>Ctenolepisma longicaudata</i> Esch.		Ba (Sr)			(Ba Sr) in dev. eggs and ♂ system
Odonata					
<i>Aeschna brevistyla</i> Ramb.					
Orthoptera					
<i>Blattella germanica</i> (L.)	Ba Sr	(Ba) caeca and ant. half MG	(Ba) middle region		Ba (Sr) } in dev. eggs and Ba Sr } male accessory glands
<i>Periplaneta americana</i> (L.)	Ba Sr	(Ba) caeca only			
Isoptera					
<i>Nasutitermes exitiosus</i> (Hill)	Ba Sr				
worker					
soldier					
<i>Coptotermes lacteus</i> Frogg.	Ba	(Ba)			
worker	Ba				
soldier					
Dermaptera					
<i>Titanolabis colossea</i> Dohrn.	Ba Sr				(Ba Sr) in dev. eggs
<i>Labidura truncata</i> Kby.	Ba				(Ba Sr) in dev. eggs
Hemiptera					
<i>Nezara viridula</i> (L.)	Ba, Sr	Ba Sr, 1st chamber			(Ba Sr) in dev. eggs
Coleoptera					
<i>Tenebrio molitor</i> L. larva					
<i>Tenebrio molitor</i> L. adult					
<i>Lyctus brunneus</i> Steph.			(Ba Sr)	(Ba Sr) near gut	
<i>Aphodius howitti</i> Hope		(Ba)			
<i>Eupacoela australasiae</i> Don.					
<i>Metorrhynchus rypidius</i> Macl.	Ba Sr	Ba Sr		(Ba Sr) near gut	Ba in dev. eggs

TABLE 2 (continued)

Insect	Malpighian Tubules	Midgut	Hindgut	Fat Body	Reproductive System
Hymenoptera					
<i>Apis mellifica</i> L.		Ba Sr			
<i>Polistes variabilis</i> Fabr.	(Ba)	Ba Sr	Ba in excretory pellets		Ba Sr in ovary, poison gland
<i>Iridomyrmex detectus</i> Sm.		Ba Sr			
<i>Camponotus consubrinus</i> (Erich.)	Ba	Ba Sr			
Neuroptera					
<i>Chrysopa</i> sp.	Ba Sr				
<i>Glenoleon pulchellus</i> Ramb.	Ba Sr	Ba Sr			
<i>Archichauliodes</i> sp.	Ba Sr				(Ba Sr) in ♂ accessory glands
Diptera					
<i>Lucilia cuprina</i> (Wied.) larva	Ba Sr restricted (Ba Sr)	(Ba) caeca and middle region MG (Ba)		(Ba)	
<i>Lucilia cuprina</i> (Wied.) adult	Ba Sr restricted				
<i>Drosophila buscki</i> Cocq. larva	Ba Sr restricted				
<i>Drosophila buscki</i> Cocq. adult					
<i>Oestrus ovis</i> L.	Ba Sr				
Lepidoptera					
<i>Ephestia kuehniella</i> Zeller larva					
<i>Tineola bisselliella</i> (Humm.) larva	(Ba)	(Ba) (Sr) ant. and post region only			Ba in dev. eggs
<i>Tineola bisselliella</i> (Humm.) adult	(Ba)	Ba Sr			
<i>Titanoceros thermoptera</i> (Low.)	(Ba)	Ba Sr			
<i>Pieris rapae</i> (L.)	Ba Sr	Ba Sr mainly post. half			Ba Sr in dev. eggs and accessory glands
<i>Heteronympha merope</i> (Fabr.)	Ba Sr	Ba (Sr)		(Ba)	Ba in lower portion of ejaculatory duct
<i>Precis villida</i> God.	Ba Sr	Ba Sr			Ba in dev. eggs

Ba—generally distinct to intense staining.

Ba—generally weak staining, occasionally no staining.

often very weak. Adult insects examined except where mentioned to the contrary.

(Ba)—only occasionally present,

and then only very occasionally and very lightly. In *Tineola* larvae the first third of the midgut generally stained lightly, the middle region did not stain at all, and the final third stained heavily (Fig. 3A) (Waterhouse, unpublished data).

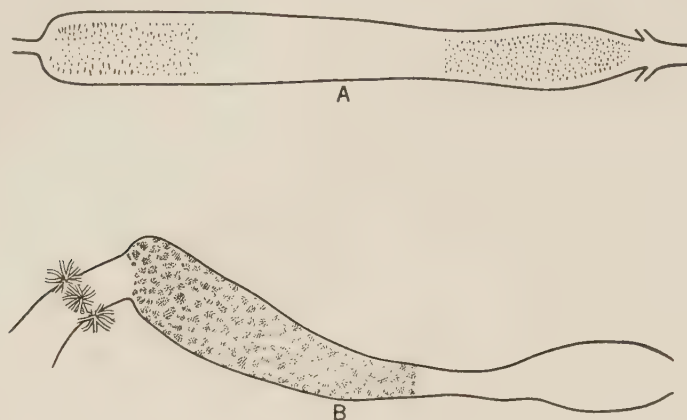


Fig. 3.—Distribution of barium and strontium in the alimentary canal. A. Midgut of *Tineola* larva. B. Hindgut of *Blattella*. Metal accumulations are indicated by stippling.

With few exceptions (see later) staining in tissues appeared to be confined to granules, the general cytoplasm remaining colourless (Plate 1, Figs. 1 to 6). However, in a few regions that stained very lightly when whole (e.g. *Periplaneta* caeca), no staining of cytoplasm or granules could be detected in sections. As a rule the nuclei remained colourless, although in some insects, e.g. *Metiorrhynchus*, *Tineola* larvae, and *Pieris*, a faint pink coloration could sometimes be seen but often only of the nucleoli.

In *Ctenolepisma* and *Heteronympha*, stained granules occur fairly uniformly in all of the mature cells of the reactive region of the midgut. In *Apis* (Plate 1, Figs. 5 and 6), *Polistes*, and *Pieris*, not all cells contain granules, which are most abundant in those cells at the apex of each fold, and less abundant or absent from the cells in the depressions. In *Apis* these granules appear to be identical with those that contain calcium (Koehler 1920) and phosphate (Day 1949). In *Tineola* larvae, feeding on cloth plus powdered yeast, the granules may either be confined to the apical zone of the cells (Plate 1, Fig. 1), or to the regenerative nidi (Plate 1, Fig. 2), or they may occur in both. These differences are presumably correlated with different stages of development of the midgut epithelium. The nucleoli occasionally stain lightly. In the adult, the granules are generally confined to the apical zone (Plate 1, Fig. 3), although occasionally some do occur at the base of the cell.

From the frequent occurrence of barium and strontium in the midgut and its usual absence in other regions of the alimentary canal it appears probable that these metals are absorbed in this segment of the digestive tract. This is confirmed by the results of feeding experiments described later.

Hindgut.—Neither barium nor strontium occur regularly in the hindgut epithelium, although staining of the contents may sometimes be seen. Thus in *Polistes*, stained food residues occur enclosed in the peritrophic membrane, indicating the direct passage through the alimentary canal of some unabsorbed barium and strontium. Weak staining of the epithelial cells was observed in the *Aphodius* hindgut and stronger staining in the middle region of the hindgut in *Blattella* (Fig. 3B). In *Blattella* this was due to granules situated most frequently between the nucleus and the lumen. These granules were most numerous in the cells at the apices of the folds (Plate 2, Figs. 2 and 3).

Other organs

Fat body.—Barium and strontium were detected in the fat body on several occasions. In some insects (e.g. *Aphodius*) some staining could be seen in the greater part of the fat body, granules being scattered throughout the multinucleate cells (Plate 2, Fig. 7). As a rule, however, staining was rather irregular in occurrence and was strictly limited to the fat body adjacent to the lower portion of the hindgut. Here the malpighian tubules commonly stain heavily and are often closely associated with the fat body. Faint staining of the nuclei could sometimes be seen (e.g. *Lucilia* larvae, *Heteronympha*).

Reproductive organs.—Barium and strontium were detected at times in both male and female reproductive organs. In the male, staining often occurred in the accessory glands (e.g. *Blattella*), although in *Heteronympha* it was absent from the accessory glands, but particularly abundant in the lower third of the ejaculatory duct. In females of most species, staining occurred in the eggs developing in the ovaries. This was often due to granular material in the nuclei of the follicular cells (e.g. *Pieris*, Plate 2, Fig. 6), but was also due to a diffuse staining of the yolk (e.g. *Heteronympha*, *Metiorrhynchus*).

(c) *Distribution of Barium and Strontium in Insects on Metal-Enriched Diets*

To supplement the information already obtained on the accumulation of deposits of barium and strontium, representative insects were fed on diets enriched with these metals. The intensity of rhodizonate staining was often greatly increased, but it was characteristic of most tissues that this increased staining was principally due to the presence of large numbers of irregular rod-shaped bodies scattered throughout the cytoplasm of the tissue or organ concerned. Under these conditions there was a far less precise localization in the cell than observed in insects on their normal diets. Strontium feeding sometimes resulted in deposits that stained more orange-red than usual.

(i) *Ctenolepisma longicaudata*.—On a metal-enriched diet the midgut of the silverfish *Ctenolepisma* stained more heavily than usual, particularly at its anterior end. Gregarines inhabiting this region were also stained. As in control insects, no staining of the malpighian tubules was observed. Silverfish fed on paper impregnated with spectroscopically pure calcium chloride stained no more heavily than controls.

(ii) *Blattella* and *Periplaneta*.—When *Blattella* were fed on 5 per cent. sugar solution containing 0.5 per cent. barium or strontium chlorides there was a considerable intensification of the staining in the middle region of the hindgut, owing to an increase in the number of stained granules near the lumen border of the cells (Plate 2, Figs. 2 and 3). The malpighian tubules also stained more intensely, particularly after barium feeding. In some individuals, the midgut caeca stained lightly and, very occasionally, barium or strontium could be detected in the rest of the midgut, particularly at the anterior end. Here the metals occur in scattered granules situated between the nuclei and the lumen. A similar picture, but with rather less intense staining, was observed with *Periplaneta* on the same diets, although it is noteworthy that no staining has been observed in the *Periplaneta* hindgut on a normal diet.

When fed 5 per cent. sucrose plus 0.5 per cent. spectroscopically pure calcium chloride, these two cockroaches seldom stained any more intensely than controls. Following treatment with chromate, the staining faded partially, but not completely, a condition similar to that of control insects. A particularly noticeable feature of calcium feeding was the absence of staining in the middle region of the hindgut, a condition that occurred so characteristically on barium and strontium diets.

(iii) *Nasutitermes exitiosus*.—When workers, soldiers, and immature reproductives of *Nasutitermes* were fed for several weeks on metal-impregnated filter paper, there was a slight increase in the staining of the malpighian tubules, but no metal uptake by the alimentary canal could be detected.

(iv) *Tenebrio molitor*.—No staining could be detected in *Tenebrio* larvae feeding on metal-enriched diets. However, both barium and strontium could be detected in the adult midgut on the same diets. Stained particles occurred throughout the cytoplasm of the inter-cryptal epithelium, particularly towards the lumen border (Plate 2, Fig. 1).

(v) *Lucilia cuprina*.—The effects of feeding metal-enriched foods to *Lucilia* larvae have already been described. Adults fed 5 per cent. sucrose solution for some days showed little staining except for a few larval granules that had not been discharged from the malpighian tubules. When fed on liver, the malpighian tubules stained more heavily, owing to the presence of granules both in the lumen and in the epithelium. The anterior and middle regions of the midgut were sometimes lightly stained, but the posterior region and the hindgut remained unstained. When fed sugar solution containing 0.1 per cent. barium or strontium chlorides, the malpighian tubules stained very intensely and the midgut generally stained strongly. The rather acid mid midgut stained most strongly, the anterior midgut, which is somewhat less acid (Waterhouse 1940), stained less strongly, and the posterior midgut, which is alkaline, stained rather weakly. On a diet of 5 per cent. sucrose plus 0.1 per cent. pure calcium chloride, both malpighian tubules and midgut sometimes stained rather more heavily than is usual for control adults. This is the only instance encountered where calcium feeding gave rise to some doubt of the absence of reaction between calcium and rhodizonate.

(vi) *Tineola bisselliella*.—When clothes moth larvae were fed on wool in which barium had been incorporated, staining was far more distinct than usual in the anterior and posterior thirds of the midgut, but absent, as before, from the middle region. Stained granules occurred in the apices of the goblet cells and, at the same level, in the columnar cells. The structureless contents of the cavities of the goblet cells often stained a uniform pink, particularly at the extreme anterior and posterior ends of the midgut. The significance of the uptake of barium and other metals by the goblet cells is discussed in detail elsewhere (Waterhouse, unpublished data). The nuclei of the staining regions often became light pink after rhodizonate treatment, the nucleoli being the only conspicuously staining elements.

(vii) *Ephestia kuhniella*.—Flour moth larvae fed on barium-enriched rolled oats showed faint staining in the midgut and heavy staining of the malpighian tubules. The latter was due to the presence of numerous stained granules in the lumen.

IV. DISCUSSION

Abundant evidence has been presented that characteristic staining of certain insect tissues follows treatment with rhodizonate. The conclusion that this staining is due to barium and strontium and not to other elements is based on the following evidence:

(1) Both barium and strontium were shown by spectrographic analyses to be present in the rhodizonate-staining portion of the malpighian tubules of *Lucilia* larvae and in heavily staining tissues of the three other insects examined.

(2) The colour of the stain produced is identical with that given by barium and strontium. Since the staining is sometimes partially or wholly discharged by chromate treatment, both elements occur in the tissues examined.

(3) The intensity of the stain increases following feeding or injection of barium and strontium, but not following similar treatment with magnesium, lead, zinc, cadmium, mercury, tin, or copper.

The evidence for absence of reaction with calcium is slightly less satisfactory. Calcium does not react under neutral conditions *in vitro* and no red staining with rhodizonate is produced following immersion of living or fixed tissues in calcium chloride solution. When calcium is fed to insects, previously fed for some time on sucrose solution only, there is, in most instances at least, no increase in staining. If we assume that calcium may on occasion interfere, the difficulty then arises that the staining observed after calcium feeding behaves to chromate as if two compounds were present, one that reacts and one that does not. It would be anticipated that any insoluble compound that a calcium salt would form with rhodizonate would not react with chromate (certainly that with calcium hydroxide does not). To fit the facts, however, we would have to postulate the formation of two different insoluble salts containing calcium and rhodizonate, these two salts having rather different solubilities as determined by their reaction to chromate.

Some evidence that calcium does not react under the conditions of test comes from the distribution of calcium in insect tissues which, when determined by buffered Gallamine blue (Stock 1949), was found to be far more widespread than that of rhodizonate staining. Tissues that stain with rhodizonate frequently also stain with Gallamine blue, but there are many tissues that stain intensely with Gallamine blue but do not react with rhodizonate (e.g. the malpighian tubules of *Apis* and the hindgut of larval and adult *Tenebrio* (see Table 2)). Furthermore, when individual insects are encountered that do not stain with rhodizonate (e.g. some *Pieris* adults), subsequent treatment with Gallamine blue produces the customary staining of the midgut. Similarly, some tissues of *Lucilia* larvae (e.g. the yellow regions of the malpighian tubules and the hindgut), which have been shown by analysis to contain appreciable quantities of calcium (Waterhouse 1950), seldom stain with rhodizonate.

Although the possibility of unusual calcium salts interfering with the rhodizonate reaction has not been entirely discounted, it can be concluded that calcium is unlikely to interfere with the staining test. However, even if other normal constituents of tissues in addition to barium and strontium are subsequently shown to produce red stains, the rhodizonate reaction will remain a valuable method for following the fate of administered barium and strontium.

As evidence against the conclusion that the rhodizonate reaction is specific for barium and strontium two objections might be advanced:

(1) The observed intensity of staining might suggest a high concentration of barium and strontium in some tissues; this was not found in spectrographic analyses (0.001 to 0.01 per cent.), and a high concentration is unlikely, particularly in view of the high toxicity of barium unless present as an insoluble salt. However, the extreme sensitivity of some histochemical tests (and the rhodizonate test is highly sensitive for barium and strontium (Feigl 1947)) is well illustrated by the staining of individual granules from the malpighian tubules of blowfly larvae. A single granule having a diameter, for example, of $6\ \mu$ and a specific gravity of 3 weighs 3×10^{-10} g. Its calcium or phosphate content is about 10 per cent. (i.e. 3×10^{-11} g.), yet granules of this size and others considerably smaller can be unmistakably stained for either element (Waterhouse 1950). It would not be surprising, therefore, if barium and strontium could be detected without difficulty in spite of their low total concentration in the granule-accumulating region of blowfly larvae. All that is required is that the local concentration should be sufficiently high to produce a visible colour with the very sensitive rhodizonate test and it is significant in this regard that, with few exceptions, only granules have been observed to stain.

(2) The feature of the present tests which, perhaps, throws greatest doubt upon the validity of the conclusion that barium and strontium are responsible for the rhodizonate staining is the widespread occurrence of the reaction in insects. However, the presence of these elements has been confirmed spectrographically. Furthermore, barium and strontium cannot be regarded as unusual body constituents since both have been reported in representatives of many

groups of animals. For example, the skeleton of the radiolarian *Podocanelius* consists almost entirely of strontium sulphate (Bütschli 1906), and barium sulphate is said to occur as granules in the cytoplasm of certain Sarcodina (Schultze 1905). Both barium and strontium have been detected spectrographically in many marine and several terrestrial invertebrates (Fox and Ramage 1931; Webb 1937) as well as in the sugar cane borer (*Rhabdocnemis*), in a cockroach (*Diploptera*), and in a centipede (*Scolopendra*), representing phytophagous, omnivorous, and predatory feeding habits (Ballard 1939). Likewise, both have been recorded in the ash of the locust *Schistocerca* (Lapp and Rohmer 1937). There are also records of the presence of these elements in mammalian tissues (Fox and Ramage 1931; Ramage and Shelton 1931; Hodges *et al.* 1950). They also occur in many plants, often in high concentrations (Webb and Fearon 1937). It may be concluded, therefore, that both barium and strontium are normal trace constituents of many tissues.

Valuable information, which fully supports the broad conclusions for staining experiments, is available from recent work with radio-active barium and strontium. For example, it has been found that ^{140}Ba is accumulated by the malpighian tubules of *Drosophila* larvae during pupation (Bowen 1948). This parallels the staining observed with rhodizonate. ^{89}Sr , absorbed by *Aedes aegypti* larvae, is not subsequently excreted by the adult. However, there is a significant loss caused by egg laying (Bugher and Taylor 1949). This indicates that strontium must pass into the developing egg, a finding that lends strong support to the validity of the rhodizonate staining observed in the developing eggs of several insects. Finally, Bowen (1948, 1949) has carried out a study of barium metabolism in hornets. ^{140}Ba was taken up by the midgut epithelium, by the genital tract, fat body, and malpighian tubules. It was absent from the nerve cord. In the midgut epithelium, ^{140}Ba was first restricted to the distal border of the cells, and considerable variations in concentration were evident. Later the concentration became more uniform, and the barium spread slowly towards the proximal border of the cells. This border, however, had not been reached by the 28th day after feeding. When high radio-activity was present in the hindgut it was found to be due to ^{140}Ba within the peritrophic membrane-enclosed faecal pellets, indicating direct passage from the midgut rather than absorption followed by excretion. Radio-activity of the fat body was evident only where it lay close to the midgut, and the tubules also had highest activity where they lay adjacent to the midgut or to the genital tract.

Bowen (1948, 1949) also obtained evidence that, after absorption by the hornet midgut, barium occurred in two chemically distinct forms. One form (probably ionic) passed rapidly from the midgut into the haemolymph and was taken up by the reproductive organs, but not by the malpighian tubules. The other form (probably a barium complex) passed more slowly into the haemolymph and was absorbed by the malpighian tubules, but never appeared in the reproductive organs. There is some indication, therefore, that hornets are well equipped to metabolize small amounts of barium.

It is clear that the radio-isotope and histochemical methods for the detection of barium and strontium are complementary. Radio-isotopes possess many advantages, such as the possibility of unequivocal detection of minute amounts, but histochemical staining is capable, under certain circumstances, of showing more accurately than radio-autographs the precise distribution of these elements in the cell, and is also valuable in demonstrating that both barium and strontium are commonly normal constituents of tissues. It is, of course, evident that the amount of barium and strontium present in the tissues depends upon the concentrations of these metals in the normal diet. It is not at all surprising, therefore, that some individuals of a species may stain intensely with rhodizonate whereas others stain little, if at all.

Little attention has been paid to the role of barium and strontium in animal metabolism and their possible importance has been almost completely overlooked. However, recent work by Rygh (1949) demonstrates that this is because diets have not been purified sufficiently to reveal the effects of their absence. Using diets purified with exceptional care, Rygh showed that small amounts of strontium and barium were indispensable to rats and guinea pigs on an otherwise adequate diet. He examined their effect on one aspect of calcium metabolism and was able to demonstrate that the calcification of bones and teeth was quite defective in the absence of strontium in spite of adequate amounts of calcium, phosphorus, and vitamin D in the diet. His work is supported by various studies in which strontium has been shown to be laid down in bone and calcified cartilage in much the same way as calcium. Although calcification is not a feature of insect metabolism, this work suggests that strontium and barium may be essential also for insects. Another explanation of their presence in insects is that the processes by which calcium is metabolized, transported, and accumulated are perhaps not sufficiently selective to exclude barium and strontium, which belong to the same subgroup of the periodic table. Certainly in some instances (e.g. the granules in the tubules of *Lucilia* larvae and in the midgut epithelium of *Apis*), calcium, barium, and strontium have been dealt with by the insect in a similar fashion, at least qualitatively.

Whatever the importance of barium and strontium may be in insects, the distribution and rather static nature of the rhodizonate-stained granules suggests that they represent accumulations held either for subsequent use or as a form of storage excretion.

V. ACKNOWLEDGMENTS

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VI. REFERENCES

- BALLARD, S. S. (1939).—Spectrographic analysis of three arthropods. *Nature* **143**: 979.
B.D.H. (1946).—"The B.D.H. Book of Organic Reagents for Analytical Use." (The British Drug Houses Ltd.: London.)

- BOWEN, V. T. (1948).—The mineral metabolism of insects. Brookhaven Conference in Biol. and Med. Report BNL-C-4: 104-8.
- BOWEN, V. T. (1949).—Barium metabolism in hornets studied by means of radioisotopes. *Trans. N.Y. Acad. Sci.* (2) 2: 68-72.
- BUCHER, J. C., and TAYLOR, M. (1949).—Radiophosphorus and radiostrontium in mosquitoes. Preliminary report. *Science* 110: 146-7.
- BÜTSCHLI, O. (1906).—Über die chemische Natur der Skeletsubstanz der Acantharia. *Zool. Anz.* 30: 784-9.
- DAY, M. F. (1949).—The distribution of alkaline phosphatase in insects. *Aust. J. Sci. Res. B* 2: 31-41.
- FEIGL, F. (1924).—Beiträge zur qualitativen Mikroanalyse II. *Mikrochemie* 2: 186-8.
- FEIGL, F. (1943).—“Laboratory Manual of Spot Tests.” (Academic Press Inc.: New York.)
- FEIGL, F. (1947).—“Qualitative Analysis by Spot Tests.” (Elsevier Pub. Co.: New York.)
- FOX, H. M., and RAMAGE, H. (1931).—A spectrographic analysis of animal tissues. *Proc. Roy. Soc. B* 108: 157-73.
- HODGES, R. M., MACDONALD, N. S., NUSBAUM, R., STEARNS, R., EZMIRLIAN, F., SPAIN, P., and McARTHUR, C. (1950).—The strontium content of human bones. *J. Biol. Chem.* 185: 519-24.
- KOEHLER, A. (1920).—Über die Einschlüsse der Epithelzellen des Bienendarms und die damit Beziehungstehenden Probleme der Verdauung. *Z. angew. Ent.* 7: 68-91.
- LAPP, C., and ROHMER, J. (1937).—Composition et valeur alimentaire du Criquet pélerin (*Schistocerca gregaria*). *Bull. Soc. Chim. Biol. Paris* 19: 321-4.
- LENNOX, F. G. (1939).—Studies of the physiology and toxicology of blowflies. 1. The development of a synthetic medium for aseptic cultivation of larvae of *Lucilia cuprina*. Coun. Sci. Industr. Res. Aust. Pamph. No. 90: 7-24.
- LENNOX, F. G. (1941).—Studies of the physiology and toxicology of blowflies. 8. Rate of ammonia production by larvae of *Lucilia cuprina* and its distribution in this insect. Coun. Sci. Industr. Res. Aust. Pamph. No. 109: 9-33.
- LILLIE, R. D. (1948).—“Histopathologic Technic.” (Blakiston Co.: Philadelphia.)
- LISON, L. (1942).—Recherches sur l'histophysiologie comparée de l'excrétion chez les arthropodes. *Mém. Acad. R. Belg. Cl. Sci.* 19: 1-107.
- NORRIS, W. P., and KISIELESKI, W. (1948).—Comparative metabolism of radium, strontium and calcium. *Symposia on Quantitative Biology* 13: 164-72.
- RAMAGE, H., and SHELTON, J. H. (1931).—Mineral content of eyes. *Nature* 128: 376.
- RYCH, O. (1949).—Recherches sur les oligo-elements. 1. De l'importance du strontium, baryum, et du zinc. *Bull. Soc. Chim. Biol., Paris* 31: 1052-61.
- SCHULTZE, F. E. (1905).—Wissenschaftliche Ergebnisse der deutschen Tiefsee Expedition II (1). (Quoted from 'Foraminifera,' Encyclopedia Britannica, 11th Ed.)
- STOCK, A. (1949).—The determination of calcium in histological sections. *J. R. Micr. Soc.* 69: 20-4.
- WATERHOUSE, D. F. (1940).—Studies of the physiology and toxicology of blowflies. 5. The hydrogen ion concentration in the alimentary canal. Coun. Sci. Industr. Aust. Pamph. No. 102: 7-27.
- WATERHOUSE, D. F. (1945).—Studies of the physiology and toxicology of blowflies. 10. A histochemical examination of the distribution of copper in *Lucilia cuprina*. Coun. Sci. Industr. Res. Aust. Bull. No. 191: 7-20.
- WATERHOUSE, D. F. (1950).—Studies of the physiology and toxicology of blowflies. 14. The composition, formation, and fate of the granules in the malpighian tubules of *Lucilia cuprina* larvae. *Aust. J. Sci. Res. B* 3: 76-112.
- WEBB, D. A. (1937).—Studies on the ultimate composition of biological material. II. Spectrographic analyses of marine invertebrates, with special reference to the chemical composition of their environment. *Sci. Proc. R. Dublin Soc.* 21: 505-39.

- WEBB, D. A., and FEARON, W. R. (1937).—Studies on the ultimate composition of biological material. Part 1. Aims, scope and methods. *Sci. Proc. R. Dublin Soc.* **21**: 487-504.
- YOE, J. H. (1938).—"A Laboratory Manual of Qualitative Analysis." (John Wiley & Sons Inc.: New York.)

EXPLANATION OF PLATES 1 AND 2

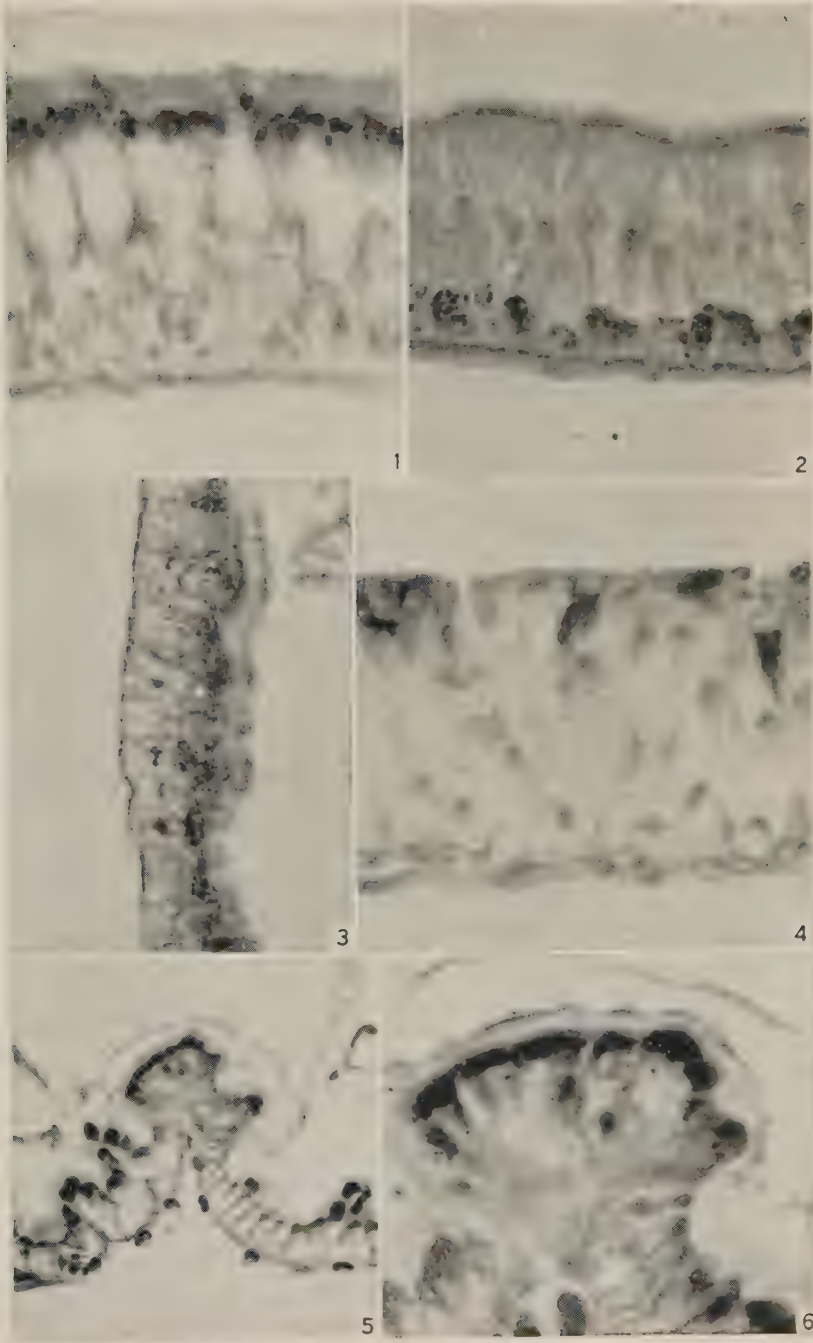
PLATE 1

- Fig. 1.—*Tineola*, L.S. larval midgut. Stained granules near the lumen borders of the columnar and goblet cells.
- Fig. 2.—*Tineola*, L.S. larval midgut. Stained granules mainly in the regenerative cells near haemocoel.
- Fig. 3.—*Tineola*, L.S. adult midgut. Granules mainly situated between nuclei and lumen.
- Fig. 4.—*Metiorrhynchus*, L.S. adult midgut. Aggregations of stained granules near lumen.
- Fig. 5.—*Apis*, L.S. adult midgut. Granules principally in cells at apex of folds.
- Fig. 6.—*Apis*. Portion of adult midgut at a higher magnification.

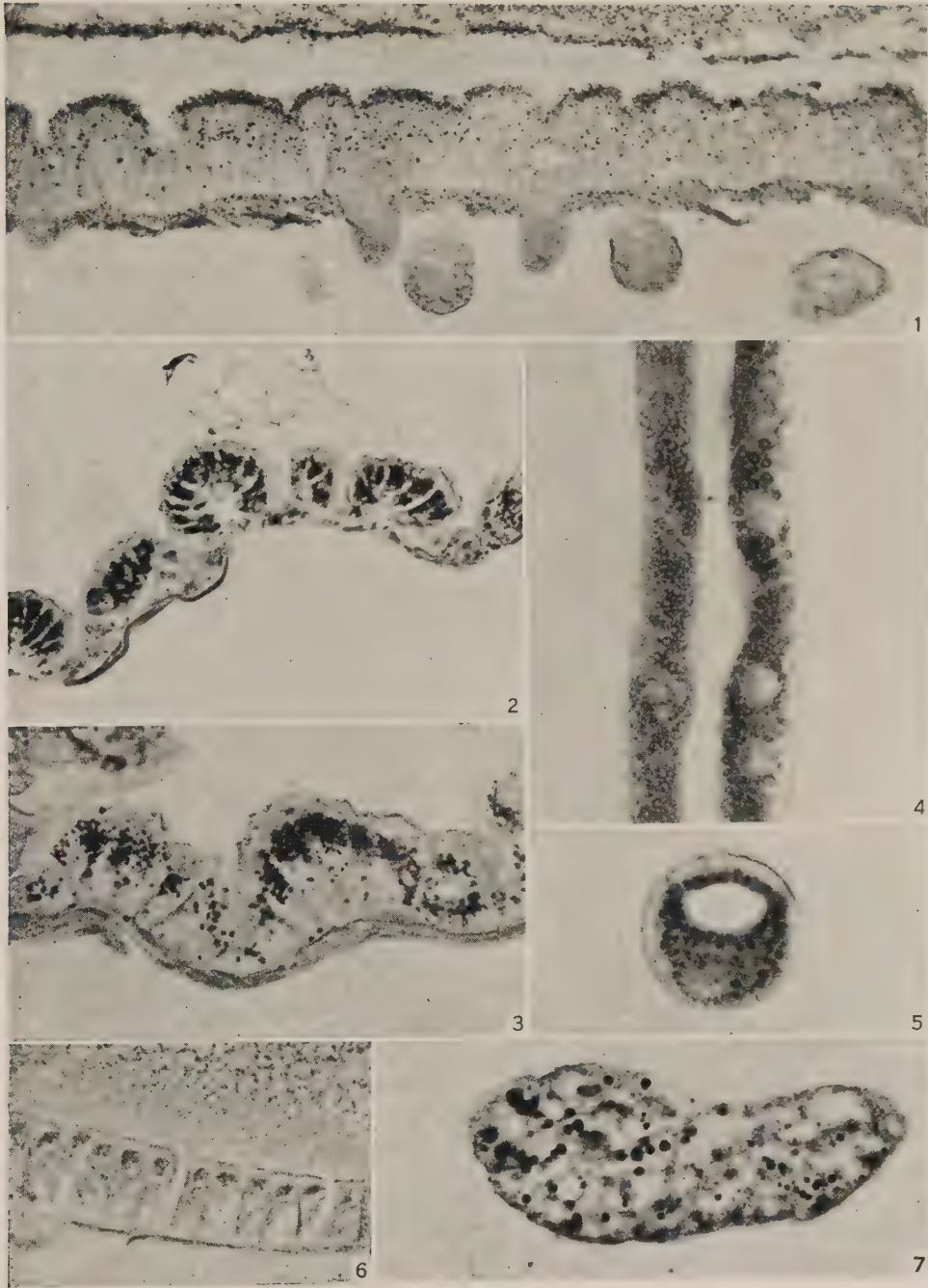
PLATE 2

- Fig. 1.—*Tenebrio*, L.S. adult midgut. Distribution of stained granules after feeding on a barium-enriched diet.
- Fig. 2.—*Blattella*, L.S. adult hindgut (middle region). Granules occupying folds of cells protruding into lumen. Barium-enriched diet.
- Fig. 3.—*Blattella*, L.S. adult hindgut (middle region). Portion of hindgut at a higher magnification, showing granular nature of stained deposits.
- Fig. 4.—*Blattella*, L.S. malpighian tubule. Stained granules, nuclei unstained.
- Fig. 5.—*Nasutitermes*, T.S. malpighian tubule. Granules absent from zone bordering haemocoel.
- Fig. 6.—*Pieris*, L.S. ovarian egg. Stained nuclei near inner borders of cells.
- Fig. 7.—*Aphodius*, T.S. fat body. Granules in a multinucleate mass of fat body.

BARIUM AND STRONTIUM IN INSECTS



BARIUM AND STRONTIUM IN INSECTS



THE MODE OF ACTION OF PHENOTHIAZINE AS AN ANTHELMINTIC. THE UPTAKE OF ³⁵S-LABELLED PHENOTHIAZINE BY THE TISSUES OF NEMATODE PARASITES AND THEIR HOSTS

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Summary

Ascaridia galli, *Nippostrongylus muris*, and *Haemonchus contortus* took up phenothiazine *in vitro* at the rate of 0.12, 0.18, and 0.26 mg./g. dry wt./hour. The uptake, which was fastest at pH 6.0, was increased in the presence of a wetting agent. It was not proportional to the relative surface area/wet weight of the parasites. The ligaturing of *A. galli* did not appreciably reduce uptake of the drug *in vitro*.

In vivo, the uptake of phenothiazine by *N. muris* and *A. galli* was about five times as fast as *in vitro*. In both parasites the drug reached a level of about 1 mg./g. dry wt. 60 minutes after dosing. Thereafter, the phenothiazine content rose steadily to a level of about 1.5-2.5 mg./g. dry wt. which was maintained for several hours. The uptake of the drug by the parasites was 5-10 times greater than that of the mucosa of the small intestine of host animals. The blood, liver, and muscle of host animals also took up the drug in much smaller amounts than did the parasites.

When *A. galli* was exposed to phenothiazine *in vitro* and *in vivo*, the concentration of the drug was found in descending order in the tissues of the intestine, the reproductive system, and muscle.

The amounts of phenothiazine found in *A. galli* expelled from the host by the action of the drug varied from 1.6 to 3.3 mg./g. dry wt.; parasites that survived treatment contained 1.1-2.1 mg./g. dry wt. Though the phenothiazine content of *N. muris* sometimes rose above 2.5 mg./g. dry wt. this parasite was not obviously affected by the drug. Both *A. galli* and *N. muris* exposed to phenothiazine *in vivo* retained a large proportion of the drug in their tissues when they were incubated in phenothiazine-free saline *in vitro*.

It is suggested that phenothiazine enters nematode parasites largely through the cuticle. The relatively small uptake of the drug by host animals as compared to their parasites may account for the differential toxicity to host and parasite. The tentative hypothesis is advanced that failure of phenothiazine to "poison" *N. muris* may be due to the fact that these parasites are not dependent on energy from anaerobic sources. The necessity for the relatively large doses of phenothiazine needed for anthelmintic treatment is discussed.

I. INTRODUCTION

In recent years the chemotherapy of bacterial and protozoal infections has improved greatly; knowledge on the mode of action of chemotherapeutic agents has been gained and the basis of the science of chemotherapy has been established. The advance in the chemotherapy of helminth infestations, however,

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has been slow. Many anthelmintics still in use are crude tissue poisons of low specificity. Among the more interesting anthelmintics is the compound thiodiphenylamine, or phenothiazine, which is used largely in the treatment of nematode infestations of the alimentary tract of the sheep. Very little precise information on the mode of action of this drug is available.

Phenothiazine has a low toxicity to host animals, and the dose rate, 0.5-1 g./kg., is high, although the solubility in water, 1 : 800,000 (Davey and Innes 1942), is low, and relatively small amounts would be needed to saturate the contents of the alimentary tract of the sheep. It is notable that a considerable proportion of the dose of phenothiazine given to a sheep is excreted unchanged in the faeces (Lipson and Gordon 1940). The range of parasites affected by the drug is limited, and its action, even on closely related species, is variable. Parasites expelled by the drug are usually alive, and live as long *in vitro* as do parasites taken from the gut of untreated animals. The nature of the anthelmintic that is effective when animals are dosed with phenothiazine is not clear. A variety of derivatives of phenothiazine are found in the alimentary tract and tissues of animals that have been dosed. There is nothing to indicate whether the anthelmintic effect is due to phenothiazine itself or to one or more of its derivatives. The present work has been carried out in an attempt to obtain some explanation of the anthelmintic action of phenothiazine.

Hotchkiss (1948) has summarized current opinion by suggesting that a proper understanding of the mode of action of bacterial chemotherapeutic agents would require such information as:

(a) The physical-chemical process by which the agent attains a toxic concentration upon or within the affected cell (simple diffusion, adsorption, active absorption);

(b) The interaction of the agent, at this concentration, with some morphological element, or with some metabolic constituent, of the cell;

(c) The interference with normal cell function occasioned by this interaction; and

(d) The alteration, gradual or immediate, in the biochemical growth processes of the cell, by virtue of this interference. In the study of the actions of anthelmintics on parasites of the alimentary tract, section (a) of Hotchkiss's conception is of particular importance. The efficiency and specificity of an anthelmintic for intestinal infestations may not necessarily depend on differences in the metabolism of the host and parasite; a drug that moves rapidly along the host's intestine may never reach a toxic concentration in the host's tissues, yet if the parasite feeds on the intestinal contents, which contain the drug, it may well absorb a toxic dose. Again, the great differences between the outer layers of the cuticle of the parasite and the absorptive surface of the host's intestine may allow the active or passive penetration of the drug into the parasite, whereas the host may absorb very little. Thus, in the study of the mode of action of phenothiazine as an anthelmintic against parasites of the alimentary tract, the relative rate of uptake of the drug by the tissues of the host and of the parasite need determination. The metabolic mechanisms

in the parasites that might be specifically antagonized by the drug can then be sought. For these reasons, the present investigation has been commenced by determining the rate of uptake and loss of phenothiazine and its derivatives by the tissues of host animals and parasites not susceptible to the action of the drug as well as of those affected by it.

A radio-active tracer has been used because it would be impossible to determine phenothiazine and its derivatives in the tissues of small parasites by conventional chemical methods. No evidence has been obtained to indicate that sulphur, or small sulphur-containing fragments, are freed from phenothiazine by the action of the biological systems studied in this investigation. Radio-active sulphur, ^{35}S , was therefore selected as a suitable tracer and the phenothiazine used has contained a known amount of the ^{35}S -labelled compound. The "phenothiazine" in tissues was determined by means of the tracer and thus included the parent compound and its sulphur-containing derivatives formed in the tissues.

Most of this work has been carried out with *Nippostrongylus muris* in the small intestine of the rat, and with *Ascaridia galli* in the small intestine of the chicken. These parasites were selected because the host animals are small and so require relatively small amounts of labelled phenothiazine, and because *N. muris* is not, whereas *A. galli* is, susceptible to the action of the drug. Further, a certain amount of information on the differences in the metabolism (Rogers 1948, 1949a; Rogers and Lazarus 1949a; Massey and Rogers 1950) and the feeding habits (Rogers and Lazarus 1949b) of these parasites is available.

II. METHODS

(a) *Biological Materials*

Nippostrongylus muris and *Ascaridia galli* were obtained from experimentally infested rats and chickens; *Haemonchus contortus* from naturally infested sheep. The parasites were separated from debris by extensive washing in normal saline. Particular care was taken to remove all contaminating radio-active material from parasites and from host-gut mucosa.

Rats, which had been fasted overnight, were lightly anaesthetized with ether before being dosed with phenothiazine, which was delivered directly into the stomach through soft catheter tubing. The chickens were also fasted before being dosed directly into the crop.

Except where stated otherwise, the labelled phenothiazine (thiodiphenylamine- ^{35}S) was diluted before use with appropriate amounts of carrier phenothiazine containing small amounts of an anionic detergent.

(b) *Synthesis of Thiodiphenylamine- ^{35}S*

The radio-active sulphur was obtained either as sulphate or sulphide. In the former instance, the sulphate, as barium sulphate, was reduced to sulphide in a hydrogen atmosphere in the presence of wood charcoal after the addition of a suitable amount of carrier. Sulphide containing ^{35}S was, if necessary,

diluted with carrier, and acidified. The hydrogen sulphide evolved was carried by a slow stream of nitrogen, trapped in potassium iodide saturated with iodine, and acidified with hydrochloric acid. Sulphur was recovered after removing the iodine with stannous chloride, purified by washing with hydrochloric acid and water, and dried in a vacuum desiccator (du Vigneaud 1944).

Phenothiazine labelled with ^{35}S was synthesized from sulphur and diphenylamine in the presence of catalytic amounts of iodine as the temperature was raised from 140 to 200°C. The hydrogen sulphide formed during the reaction was removed in a slow stream of nitrogen and trapped in the iodine-potassium iodide solution as before. Thus, the ^{35}S not utilized for phenothiazine synthesis was recovered. The melting point of the impure reaction product was usually about 175°C.

Purified phenothiazine was obtained by sublimation at about 260°C. and 25 mm. pressure. This material, prepared on a number of occasions, consisted of pale gold or faint green crystals and always gave melting points in the region of 182°C. Melting points from 180°C. (Lange 1939) to 185°C. (Smith and Nelson 1942) have been assigned to phenothiazine by different workers.

(c) Preparation of Samples for Assay of Radio-activity

Tissue samples, approximately 50 mg. wet weight, were quickly dried on filter paper and weighed. It was found that the sulphur in such amounts of tissue could be suitably oxidized by the micro-Carius method (Niederl *et al.* 1940), 0.25 ml. of concentrated nitric acid and 5 mg. sodium bromide being used per sample. Digestions were carried out for 2 hours at 100°C. followed by 20 minutes at 300°C.

Following digestion, the cooled micro-Carius tubes were opened at the tip and the contents allowed to drain to the blunt end of the tube. The top of the micro-Carius tube was then cut off, leaving the digest in a tube of suitable size and shape for the subsequent manipulations. Nitric acid was removed from the digest under reduced pressure at 100°C. The material on the top portion of the micro-Carius tube was washed into the lower portion with 3 ml. of distilled water. The sulphate was then precipitated as the barium salt in the usual way, concentrated by centrifuging, transferred in water to standard glass counting dishes, and dried at 100°C. This procedure gave an even distribution of sulphate over the well in the counting dish.

(d) Determination of Radio-active Sulphur

^{35}S was determined by means of a thin mica-window Geiger-Müller tube driving a scale-of-eight. The geometrical relation of sample to tube window was constant within small limits; corrections for resolving time and self-absorption were made empirically.

A sufficient number of counts was taken, usually 20×1 minute counts, alternating with 1 minute background counts so that the range of variation from the mean was usually less than ± 10 per cent. The higher concentrations

of ^{35}S were counted for appropriately shorter periods. All radio-activity measurements were relative in that no corrections were made for the radiation that did not penetrate the sensitive volume of the Geiger-Müller tube.

An empirical standard for equating β -radiation with weight of phenothiazine was prepared by dissolving known amounts of phenothiazine containing the labelled compound in alcohol and determining the radio-activity of a suitable aliquot that had been dried on a counting dish at 70°C . No appreciable error was caused by using this procedure rather than converting the phenothiazine- ^{35}S to barium sulphate- ^{35}S for counting. A barium sulphate- ^{35}S standard was used for testing variations in the sensitivity of the Geiger-Müller counter. Suitable corrections for ^{35}S decay were made when necessary.

III. PROCEDURE AND RESULTS

(a) Uptake of Phenothiazine *in vitro*

Parasites, washed free of host ingesta and mucus, were incubated at 37°C . in normal saline, held at pH 4.5 with 0.04M acetate buffer for *Haemonchus contortus*, or at pH 6.8 with 0.05M phosphate buffer for *Nippostrongylus muris* and *Ascaridia galli*. The hydrogen ion concentrations were selected as being similar to those of the *in vivo* environments of the respective parasites. Dissolved and suspended labelled phenothiazine, 2 mg./ml., stabilized with traces of wetting agent, a dialkyl-naphthalene sulphonate, was present in the media. The ^{35}S content was in the region of 2×10^{-4} $\mu\text{c.}/\text{ml}$.

TABLE 1
UPTAKE OF PHENOTHIAZINE BY NEMATODE PARASITES *IN VITRO* AND *IN VIVO*

Species	Uptake <i>in vitro</i> (mg./g. dry wt.)	Uptake <i>in vivo</i> (mg./g. dry wt.)	Relative Surface Area/Unit Wt.
<i>Ascaridia galli</i>	0.21-0.32 (0.24, 4)	0.41-1.76 (1.12, 4)	1
<i>Nippostrongylus muris</i>	0.32-0.40 (0.37, 3)	0.53-2.50 (1.70, 4)	19
<i>Haemonchus contortus</i>	0.52-0.54 (0.53, 2)	—	11

The figures in brackets show the mean value of the phenothiazine uptake and, in italics, the number of experiments carried out.

At intervals, parasites were taken from the media and the uptake of phenothiazine determined by estimating their ^{35}S content. The results are shown in Figure 1. The uptake of the drug *in vitro* and *in vivo* over a period of 2 hours are compared in Table 1. The experiments carried out *in vivo* will be described in a later section of this paper.

The relative surface areas per unit weight were calculated by treating the parasites as long, uniform cylinders. The diameters used in the calculations were average values, obtained by measuring a representative number of the different parasites used in the experiments at three points along their lengths.

(b) *Effect of Ligaturing Ascaridia galli on the Uptake of Phenothiazine in Vitro*

A. galli was exposed to labelled phenothiazine under conditions similar to those of the previous experiments carried out *in vitro*, except that no wetting agent was used. Some male specimens were tightly ligatured at the head and tail to prevent the entry of the drug through the orifices of the alimentary tract or sex organs. At intervals, parasites were taken from the medium,

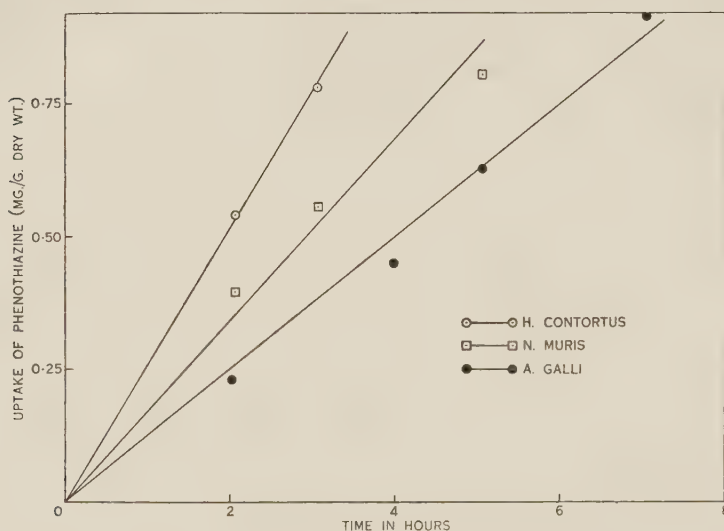


Fig. 1.—Uptake of phenothiazine *in vitro* by *Haemonchus contortus*, *Nippostrongylus muris*, and *Ascaridia galli*. The parasites were exposed to labelled phenothiazine in saline buffered at pH 4.5 for *H. contortus*, and pH 6.8 for the other parasites. A wetting agent was present in the saline.

washed, the ligatures removed and the ^{35}S content determined. The results of a typical experiment are given in Table 2. Four parasites were examined after being exposed to the drug for 2 hours, and two were examined after 3½ and 5 hours exposure respectively. It was found that ligaturing only slightly reduced the rate of drug uptake.

TABLE 2
UPTAKE OF PHENOTHIAZINE BY LIGATURED AND UNLIGATURED *ASCARIDIA GALLI* MALES *IN VITRO*

Condition of the Parasites	Phenothiazine Uptake (mg./g. dry wt.)		
	2 Hours	3½ Hours	5 Hours
Ligatured	0.14, 0.16	0.22	0.34
Unligatured	0.14, 0.18	0.30	0.39

As *A. galli* probably did not feed on the medium *in vitro*, the failure of ligaturing to cause an appreciable reduction in the uptake of phenothiazine

was not surprising. Thus, although the possibility that *A. galli* may *in vivo* take up phenothiazine *per os* cannot be precluded, it is clear that, under the conditions of these experiments, the cuticle of the parasites was penetrated by the drug.

Over a long series of experiments, no consistent difference was observed in the rate of uptake of phenothiazine by male and female parasites.

(c) *Effects of pH and Wetting Agent on the Uptake of Phenothiazine in vitro*

A. galli was exposed, at 37°C., to ³⁵S-labelled phenothiazine in saline containing 0.05M phosphate buffer at pH 5.3, 6.0, 6.6, and 7.3, with and without traces of the wetting agent, sodium dialkyl-naphthalene sulphonate. The phenothiazine content of the parasites was determined after they had been exposed to the drug for 2, 4, 5, and 6½ hours. Typical results are shown in Table 3. It was found that the rate of uptake per hour under given conditions was almost constant over the period of time examined. The wetting agent increased uptake at all the hydrogen ion concentrations used. A slight peak in the rate of uptake was obtained at pH 6.0.

TABLE 3
PHENOTHIAZINE UPTAKE IN *ASCARIDIA GALLI* AT DIFFERENT HYDROGEN ION
CONCENTRATIONS IN THE PRESENCE AND ABSENCE OF WETTING AGENT

pH	Phenothiazine Uptake (mg./g. dry wt./hr.)	
	Buffered Saline	Buffered Saline plus Wetting Agent
5.3	32	95
6.0	55	135
6.6	45	135
7.3	36	110

(d) *Distribution of Phenothiazine in the Tissues of Ascaridia galli*

A. galli was the only species of parasite used in this investigation that was large enough to allow satisfactory dissection and separation of the different organs and tissues for ³⁵S determination. After exposure at 37°C. to labelled phenothiazine for 4 and 28 hours in saline buffered at pH 6.0 and containing a little wetting agent, the organs of the parasites were separated by dissection, weighed, and the ³⁵S content determined. The relative amounts of phenothiazine in the cuticle, reproductive system, and intestine are shown in Figure 2, A and B. The distribution of phenothiazine in the tissues of parasites exposed to the drug for about 3 hours *in vivo* is shown in Figure 2C.

It should be noted that the muscle layers were included with the cuticle. The lumen of the intestine was not washed and so may have contained ingested but unabsorbed phenothiazine in the parasites exposed to the drug *in vivo*. In the other experiments it was unlikely that unabsorbed drug was present, be-

cause, as already shown, the drug was not taken up by the parasites *per os*. "Ovary," mentioned in Figure 2, refers to the whole female reproductive system and includes the ovary, uterus, and its contained eggs.

(e) *Uptake of Phenothiazine by Nippostrongylus muris and Ascaridia galli in vivo*

A necessary preliminary to these experiments was the determination of the rate of passage of phenothiazine along the alimentary tracts of the experimental animals. It was found that phenothiazine, given directly into the stomach of a fasted rat that had been lightly anaesthetized with ether, appeared in that portion of the small intestine normally inhabited by *N. muris* (11-26 cm. from the pylorus) 15-20 minutes after dosing. Phenothiazine reached the positions in the small intestine in which *A. galli* was normally found $\frac{1}{2}$ -1 $\frac{1}{2}$ hours after the drug had been delivered into the crop.

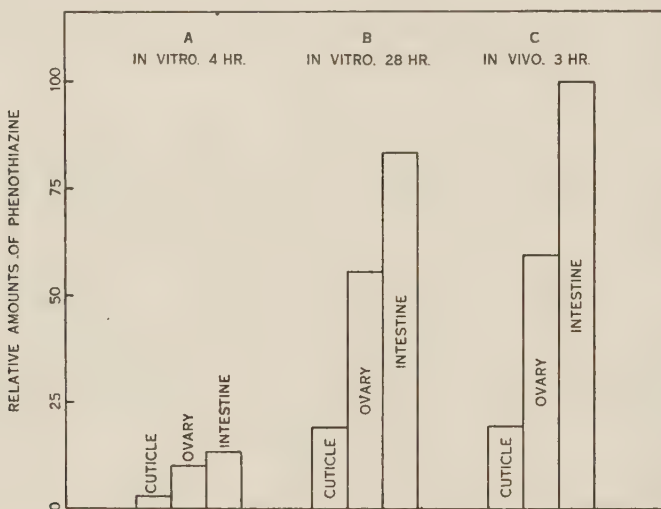


Fig. 2.—Distribution of phenothiazine in the tissues of *Ascaridia galli* exposed to the drug *in vitro*, A, for 4 hours, B, for 28 hours, and *in vivo*, C, for 3 hours. The muscle was included with the "cuticle"; "ovary" refers to the whole female reproductive system and includes the ovary, uterus, and its contained eggs.

These figures were used when calculating the period for which parasites were exposed to phenothiazine *in vivo*. Thus the *A. galli* used for the experiments carried out *in vivo*, and referred to in Table 1, were taken from chickens 3 hours after they had been dosed. The *N. muris* were used 2 $\frac{1}{2}$ hours after dosing. In both instances the parasites were exposed to the drug for about 2 hours.

To determine the rate of uptake of phenothiazine *in vivo* by *N. muris*, heavily infested rats weighing 100-120 g. were fasted overnight and then dosed, 1 g./kg., with phenothiazine containing 5-10 μ c. of the ^{35}S -labelled compound.

Each dose was given in about 2 ml. of water, and was stabilized with small amounts of an anionic detergent. At intervals after being dosed, animals were killed and parasites collected. Samples of blood were collected; liver, gut mucosa, and intercostal muscle were also taken from the host animals, washed quickly in saline, and excess moisture was removed with filter paper. The tissues were then weighed and their ^{35}S contents determined. The samples of gut mucosa were taken from the small intestine at distances of 10, 18, and 26 cm. from the pylorus. The mucosa was dissected from the gut wall and freed of mucus before being washed and prepared for ^{35}S determination. The three gut samples were digested together in the one micro-Carius tube. The several sites for taking the gut-mucosa samples were selected as being most appropriate for obtaining the relative rates of uptake of phenothiazine by parasites and by host gut. Care was taken to avoid contaminating the samples of host tissue with the radio-active phenothiazine in the gut contents. The parasites and host-gut mucosa were carefully and repeatedly washed to ensure that all extraneous phenothiazine was removed.

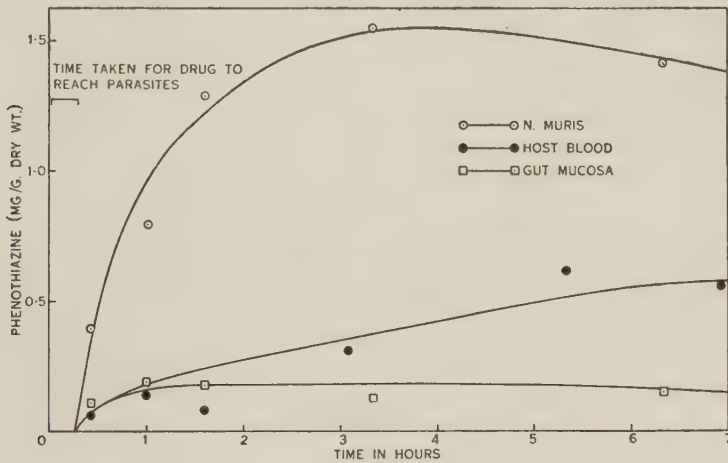


Fig. 3.—Uptake of phenothiazine by *Nippostrongylus muris* *in vivo*, by the blood of the host, and by the mucosa of the small intestine of the host. The samples for the estimation of uptake in the gut mucosa were taken 10, 18, and 26 cm. from the pylorus. For convenience, the uptake of phenothiazine by parasites and by the tissues of the hosts is shown as commencing at the same time; in fact, it is probable that the tissues of the hosts commenced taking up the drug somewhat before the parasites.

One set of results showing the uptake of phenothiazine by *Nippostrongylus muris*, as well as by the gut mucosa and the blood of host animals, is shown in Figure 3. The amount of phenothiazine taken up by the liver was greater than that found in gut mucosa but less than that in blood. It reached a steady level of about 0.3 mg./g. some 75 minutes after the dosing. Drug uptake by intercostal muscle was lower than that of the gut mucosa; it rose to a value of about 0.1 mg./g. dry wt. four hours after the dosing.

The results obtained in different experiments varied somewhat. Thus, on occasions, there was a peak in the uptake of phenothiazine by the parasites about three hours after the dosing, the level sometimes nearly reaching 3 mg./g. dry wt. The very steep rise in uptake found 20 minutes after the dosing, or some 5 minutes after the drug had reached the parasites, was found in all experiments. Sometimes a small peak in the amount of phenothiazine in the blood and muscle of host animals was also found at this time.

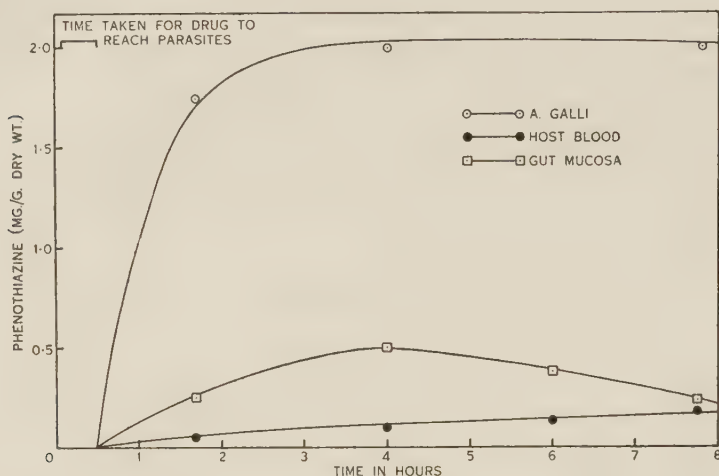


Fig. 4.—Uptake of phenothiazine by *Ascaridia galli* *in vivo*, by the blood of the host, and by the mucosa of the small intestine of the host. The samples for determining the uptake by the gut of the host were taken 30 and 46 cm. from the pylorus. For convenience, the uptake of phenothiazine by parasites and by tissues of the hosts is shown as commencing at the same time; in fact, it is probable that the tissues of the hosts commenced taking up the drug somewhat earlier than the parasites.

To determine the uptake of phenothiazine by *A. galli* and the tissues of its host, infested chickens that had been fasted overnight were dosed with appropriate amounts of drug containing 2-3 $\mu\text{c.}$ of ^{35}S -labelled material. Birds were killed at intervals and the ^{35}S in individual parasites as well as in the mucosa of the hosts' gut, liver, blood, and intercostal muscle was determined. The samples of gut mucosa were taken from the small intestine, 30 and 46 cm. from the pylorus; the two samples were analysed together. Figure 4, taken from the results of a typical experiment, shows the amount of drug found in the parasites as well as in the blood and gut mucosa of host animals. The points showing uptake by the parasites are averages calculated from the amounts found in individual parasites in each bird. The variation among the parasites from the bird killed 1½ hours after dosing was ± 24 per cent. The variation in the parasites from birds killed later in the experiments, though large, was less than this. The difference in the amount of phenothiazine found

in several parasites from the same bird was probably partly due to their varied positions in the host gut causing a difference in the time of exposure of individual parasites to the action of the drug.

In determining the uptake of phenothiazine by *N. muris*, as shown in Figure 3, large numbers of these small parasites were used for each ^{35}S determination. The points on the graph, therefore, represent the average uptake of as many as 500 parasites. *N. muris* was always confined to a relatively small region of the host intestine and there were probably no great differences in the times for which individual parasites were exposed to the drug except during the early parts of the experiments.

(f) *Concentration of Phenothiazine in the Tissues of Ascaridia galli Necessary for Anthelmintic Action*

This section of the investigation was concerned with an attempt to determine the minimum amount of phenothiazine, taken up *in vivo* by *A. galli*, that would result in the expulsion of the parasites from the host. *N. muris* was not appreciably affected by the anthelmintic and was never expelled from host animals by the action of the drug.

The minimum time that elapsed after the dosing of a chicken before parasites were passed out in the faeces was about 6 hours. For a further 24 hours parasites were expelled by the action of the drug. Thus, when chickens were killed less than 30 hours after treatment, it was necessary to distinguish between parasites passing out of the host and those that would survive treatment. Experience showed that any parasite more than half the length of the small intestine from the pylorus could be considered to be passing out of the host. Using this criterion, it was possible to select parasites that had survived anthelmintic treatment in treated chickens and those that had been, or would be, expelled by the action of the drug.

TABLE 4
PHENOTHIAZINE IN PARASITES AFTER EXPOSURE TO THE DRUG *IN VIVO*

<i>Ascaridia galli</i>	Phenothiazine in Parasites (mg./g. dry wt.)		
	Surviving Treatment	Leaving the Host	Expelled from the Host
Males	1.1 (—, 1)	1.9 (1.6-2.9, 4)	2.3 (1.6-3.0, 3)
Females	1.6 (1.1-2.1, 2)	2.2 (1.4-3.2, 15)	2.6 (2.0-3.3, 5)

The figures in brackets show the limits of variation in the amounts of phenothiazine found in individual parasites, and, in italics, the number of parasites examined.

Chickens infected with *A. galli* were dosed in the usual way; the parasites were collected as they were expelled and were prepared for ^{35}S determination. After periods of 6-7½ hours the chickens were killed, the small intestine was opened, the positions of the parasites were noted, and specimens were taken for ^{35}S determination. In Table 4 the results are shown as the amounts of phenothiazine found in (i) parasites that survived treatment, (ii) parasites that

could be regarded as passing out of the host, and (iii) parasites expelled from the host. The figures in the table show the average amounts of phenothiazine found; the figures within brackets show the limits of variation in the amounts of phenothiazine found in different parasites and the numbers of parasites examined. Although the average amount of drug in the groups (i), (ii), and (iii) steadily increased, there was considerable overlapping in the results and it is difficult to say at what level phenothiazine reached a "toxic" concentration in the parasites. Evidently the toxicity varied greatly under different conditions; at times 1.5 mg./g. dry wt. was "toxic," on other occasions 2 mg. was not sufficient to cause the expulsion of the parasites. It might appear that the parasites that took up most phenothiazine were expelled from the host first, but again this was not a consistent finding. The results suggest that, in general, male parasites were affected by smaller amounts of the drug than were female parasites, a fact associated, perhaps, with the relatively greater surface area per unit weight of the male parasites.

TABLE 5
RETENTION OF PHENOTHIAZINE IN PARASITES EXPOSED TO THE DRUG *IN VIVO*

<i>Ascaridia galli</i>		<i>Nippostrongylus muris</i>	
Unincubated	Incubated	Unincubated	Incubated
2.2	1.7	0.9	0.9
1.5	1.7	1.5	0.9
1.4	2.0	0.8	1.0
1.6	1.8	0.9	1.2
2.2	2.6	1.1	1.0
		0.9	0.9
		1.0	0.6

Parasites were taken for the immediate determination of phenothiazine and for determination after incubation in phenothiazine-free saline for 24 hours at 37°C. Each pair of results was obtained from a separate experiment. The results are given in mg./g. dry wt.

(g) *Retention of Phenothiazine in Parasites Exposed to the Drug in vivo*

Infected animals were killed at different times after they had been dosed with appropriate amounts of labelled phenothiazine, and the parasites were collected. *N. muris* from one or two rats were mixed together, washed, and divided into two portions. One lot was used immediately for ^{35}S determination, the other lot was incubated at 37°C. in phenothiazine-free saline before the ^{35}S determinations were made. To examine the retention of phenothiazine in *A. galli*, parasites that had been exposed to the anthelmintic in the same host and found in the same section of the host's intestine, were used. Some of the parasites were incubated for 24 hours before the ^{35}S was determined, others were used immediately. The results obtained are shown in Table 5. With *A. galli*, males were compared with males, and females with females; the samples of *N. muris* contained both females and males. It should be noted that the

parasites taken from treated animals and incubated *in vitro* remained alive and active for the same time, as far as could be judged, as control parasites that had not been exposed to the anthelmintic. Even parasites expelled from treated birds were as active and long-lived *in vitro* as were normal *Ascaridia galli*.

IV. DISCUSSION

The experiments carried out in this investigation did not show definitely whether the major route of entry of phenothiazine into the parasites was *per os* or through the cuticle. The arguments in support of the former suggestion are:

(a) The rate of uptake *in vitro* was not proportional to the relative surface area/unit weight of the parasites (see Table 1);

(b) Uptake *in vivo*, when the parasites might be expected to be feeding, was more rapid than *in vitro* (see Table 1); and

(c) Relatively larger amounts of phenothiazine were found in the intestine of *A. galli* when it was exposed to the drug *in vivo* (see Fig. 2).

In favour of entry via the cuticle are the observations that:

(d) The ligaturing of *A. galli* did not prevent the entry of the drug into the parasites *in vitro*;

(e) The uptake of drug by *N. muris*, which feeds on host tissue (Rogers and Lazarus 1949*b*), was of the same order as that of *A. galli*, which feeds on the host's gut contents. Further, phenothiazine is known to be active against *Ascaris lumbricoides*, which feeds largely on ingesta (Rogers 1940*a*), and against *Strongylus* spp., which feed on the tissue of the host (Rogers 1940*b*). Sheep parasites of the genus *Trichostrongylus*, and *Haemonchus contortus*, which are affected by the anthelmintic, feed on the tissue of the host (Esserman and Sambell, personal communication). Thus, it appears that the uptake of phenothiazine by a large variety of nematode parasites is independent of their feeding habits. As relatively small amounts of phenothiazine were found in the tissues of the rat and the chicken, it seems reasonable to suppose that the amounts taken up by other host animals would be small. Hence, parasites which feed on their host's tissues could only obtain small amounts of the drug with the materials they ingest.

(f) The particle size of phenothiazine administered to sheep does not greatly affect its efficiency (Gordon 1940). If solid particles of the drug were ingested by the parasites it would be expected that smaller-sized preparations would be considerably more effective.

(g) Lastly, Esserman (personal communication) has found that ³⁵S-labelled phenothiazine is taken up more rapidly via the cuticle than via the intestine of *Ascaris lumbricoides*, even when the drug is injected directly into the lumen of the parasite's intestine. It would appear, therefore, that uptake through the cuticle of the parasites was the major route of entry of the drug and some explanation is required of the apparently conflicting results (a), (b), and (c) mentioned above.

The disproportionality between drug uptake *in vitro* and the relative surface areas of the parasites might have depended on difference in the affinity for the drug of the outer cuticular surfaces of the different species. *Haemonchus contortus*, which lives in the highly acid medium of the abomasum of the sheep, might be expected to differ considerably from the other parasites in this respect. The faster rate of uptake of phenothiazine *in vivo* could have been due to several factors other than the feeding habits of the parasites. The physical and chemical conditions in the gut might have caused the formation of derivatives of phenothiazine or complexes of phenothiazine with materials in the gut contents which penetrated the cuticle more rapidly than phenothiazine itself. It appears that hydrogen ion concentration over a wide range did not greatly affect phenothiazine uptake and it seems likely that the ionization of the drug itself, or of the molecules on the surface of the parasites, was not of major importance in determining the efficiency of the drug in penetrating the cuticle.

The amount of phenothiazine taken up by the parasites *in vivo* was very much greater than that taken up by the host animals (see Figs. 3 and 4) and it appears that there is no necessity to postulate a metabolic difference between the hosts and the parasites to account for differences in their susceptibility to the toxic effects of the drug. Taking into consideration the highly convoluted nature of the mucosa of the small intestine, the differences in uptake between host and parasite cannot be entirely attributed to the relative areas of the absorbing surfaces per unit weight. The cuticle of nematode parasites is largely albuminoid (Chitwood 1936), probably with a fatty surface layer (Trim 1949), and differs greatly from the mucosa of the small intestine of the host. It is possible, then, that the surface of the parasites has a greater affinity for or is more easily permeated by phenothiazine than is that of the host's intestine. This is suggested by the very sudden rise in the phenothiazine content of the parasites, almost as soon as the drug reached them *in vivo*.

The amount of phenothiazine taken up by *N. muris*, which was not obviously affected by the drug, was frequently as great as that taken up by the susceptible parasite, *A. galli*. The failure of phenothiazine to affect the one parasite but not the other may have been due to the formation of toxic derivatives in the intestine of the chicken but not in that of the rat, or to differences in the metabolism of the two parasites. Some information bearing on the latter question is available and may be discussed here.

A. galli and *N. muris* both respire actively *in vitro* when oxygen is available (Rogers 1948) and both can obtain energy by oxidative mechanisms similar to the tricarboxylic acid cycle (Massey and Rogers 1950). However, *in vivo*, though sufficient oxygen is available to allow *N. muris* to respire actively, *A. galli* is probably anaerobic (Rogers 1949a, 1949b). As far as is known at present, this is the major metabolic difference between the parasites as they exist *in vivo*. It is possible, then, that the efficiency of phenothiazine as an anthelmintic is related to the dependence of parasites on anaerobic energy sources except in large parasites, which, though anaerobic, have a relatively small surface area over which phenothiazine absorption might take place.

The retention of phenothiazine in the tissues of *A. galli* that had been exposed to the drug was very strong (see Table 5). It appears that the ability of the parasite to excrete the drug, when incubated *in vitro*, was poor. One result obtained from a parasite that had survived treatment with phenothiazine and was found in the chicken 24 hours after dosing, showed that the retention probably occurred *in vivo* also. The parasite contained 0.6 mg. phenothiazine/g. dry weight; the small intestine of the host contained no detectable phenothiazine. It is surprising that *Ascaridia galli* that had been expelled from host animals by the action of the anthelmintic should remain as active as untreated parasites, and for as long a period, when incubated in saline at 37°C. The results of these experiments suggest that attempts to assess the activity of phenothiazine or its derivatives *in vitro*, based on survival time of the parasites, would be of little value. However, it has been found that parasites "poisoned" with phenothiazine lost much of their egg-laying activity and this was not recovered when they were incubated in phenothiazine-free saline (see Fig. 5).

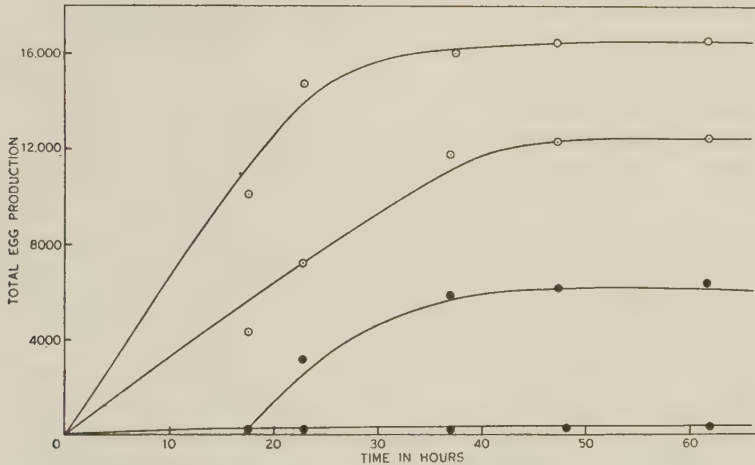


Fig. 5.—Effect of phenothiazine on the egg production of *Ascaridia galli*. The two upper curves show the egg production of normal parasites *in vitro*; the two lower curves show the results obtained from parasites exposed to phenothiazine *in vivo* before they were incubated *in vitro*.

On some occasions, phenothiazine was lost from the tissues of *Nippostrongylus muris* (see Table 5) when the treated parasites were incubated *in vitro*. It might be thought that this could have been due to a failure to obtain representative samples of parasites for assaying the phenothiazine content before and after incubation. However, the results obtained when samples from one lot of parasites that had been treated with the drug were analysed without incubation did not show great variation. For example, in one experiment, three such groups of determinations showed variations of less than ± 10 per cent. The variable retention in *N. muris* may have been due to the fact that these parasites became inactive, and presumably died, much more quickly *in*

vitro than *A. galli*. Death of *N. muris* was associated with changes in the permeability of the cuticle because haemoglobin was freed into the medium from parasites that had been incubated for periods greater than 30 hours. It is probable, then, that the irregular losses of phenothiazine were due to changes in cuticle caused by the death of some of the parasites.

The survival of *A. galli*, which retained "toxic" amounts of phenothiazine when incubated in saline *in vitro*, may have been due to the drug acting on anaerobic mechanisms only, and, as oxygen was available to the parasites *in vitro*, they were able to obtain sufficient energy from oxidative mechanisms. Unfortunately, *A. galli* becomes inactive when incubated anaerobically *in vitro* and it is difficult to assess the action of phenothiazine on survival time.

The amount of phenothiazine required to have a "toxic" action on parasites *in vivo* was large relative to the amount of phenothiazine present in solution in the contents of the host gut. The small intestine of a fasted chicken would probably contain less than 10 ml. of fluid, which, when saturated with phenothiazine, would hold less than 13 μ g. of the drug. About 250 μ g. would be required for ten adult *A. galli* before a "toxic" concentration of drug in the parasite tissues was reached. Thus, all the drug in solution would be used up some 15 times before the parasites would become poisoned, even if the amount of drug taken up by the host is not considered. It would be necessary, then, to have a large excess of solid phenothiazine present to maintain an adequate concentration of drug in solution in the contents of the host's gut along the length of the small intestine long enough for the drug to reach a "toxic" concentration in the parasites. This may be at least a partial explanation of the large dose rate of phenothiazine required for its action as an anthelmintic.

The present investigation has given rise to several questions. Do the derivatives of phenothiazine that occur in the gut contents of the rat and chicken, and in the tissues of *N. muris* and *A. galli*, differ? Has the drug a high affinity for the nematode cuticle? Does it function by preventing the parasites from obtaining energy from anaerobic sources? These questions arise from the consideration of hypotheses which admittedly have slender foundations; nevertheless, the answers will undoubtedly assist in obtaining an understanding of the mode of action of phenothiazine as an anthelmintic.

V. ACKNOWLEDGMENTS

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VI. REFERENCES

- CHITWOOD, B. G. (1936).—*Proc. Helminth. Soc. Wash.* 3: 1.
- DAVEY, D. G., and INNES, J. R. M. (1942).—*Vet. Bull.* 12: 401.
- GORDON, H. McL. (1940).—*J. Coun. Sci. Industr. Res. Aust.* 13: 85.
- HOTCHKISS, R. D. (1948).—*Ann. Rev. Microbiol.* 2: 183.
- LANGE, N. A. (1945).—"Handbook of Chemistry." (Handbook Publishers Inc.: Ohio.)
- LIPSON, M., and GORDON, H. McL. (1940).—*J. Coun. Sci. Industr. Res. Aust.* 13: 172.
- MASSEY, V., and ROGERS, W. P. (1950).—*Aust. J. Sci. Res. B* 3: 251.
- NIEDERL, J. B., BAVIN, H., MCCOY, J. S., and KUCK, J. A. (1940).—*Industr. Engng. Chem.* 12: 428.
- ROGERS, W. P. (1940a).—*J. Helminth.* 18: 53.
- ROGERS, W. P. (1940b).—*J. Helminth.* 18: 103.
- ROGERS, W. P. (1948).—*Parasitology* 39: 105.
- ROGERS, W. P. (1949a).—*Aust. J. Sci. Res. B* 2: 166.
- ROGERS, W. P. (1949b).—*Aust. J. Sci. Res. B* 2: 157.
- ROGERS, W. P., and LAZARUS, M. (1949a).—*Parasitology* 39: 302.
- ROGERS, W. P., and LAZARUS, M. (1949b).—*Parasitology* 39: 245.
- SMITH, L. E., and NELSON, O. A. (1942).—*J. Amer. Chem. Soc.* 64: 461.
- TRIM, A. R. (1949).—*Parasitology* 39: 281.
- DU VICNEAUD, V. (1944).—*J. Biol. Chem.* 154: 249.

SEPARATION OF SATURATED MONO-HYDROXAMIC ACIDS BY PARTITION CHROMATOGRAPHY ON PAPER

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Summary

Separation of simple saturated hydroxamic acids with a chain length of one to nine carbon atoms has been achieved by partition chromatography on paper. The relative merits of a number of solvents are discussed.

It is shown that efficient humidification of the system is important when using a solvent such as benzene, which dissolves very little water. Interference caused by metals in the filter paper can be overcome by preliminary treatment.

I. INTRODUCTION

This paper is concerned with the separation by partition chromatography on paper of the lower simple saturated hydroxamic acids ($C_nH_{2n+1}CO.NHOH$). The technique was developed for use in an investigation of volatile substances produced by fresh apples.

Carboxylic acids (both free and esterified), primary alcohols, and aldehydes can all be converted to hydroxamic acids and the separated hydroxamic acids can readily be detected as the coloured iron complexes.

After the methods of chromatographic separation described in this paper had been worked out, the work of Fink and Fink (1949) came to our notice. These authors obtained useful separations of hydroxamic acids derived from simple monocarboxylic acids with one to five carbon atoms; above this, their series was incomplete and not well separated. They also studied hydroxamic acids from a number of substituted and polycarboxylic acids.

The principles on which the paper chromatographic technique is based have been adequately discussed by Consden, Gordon, and Martin (1944) and, with respect to the separation of acids, by Lugg and Overell (1948). The technique of ascending chromatography, as described by Williams and Kirby (1948), has been used in the present investigations.

II. DEVELOPMENT OF TECHNIQUE FOR CHROMATOGRAPHIC SEPARATION OF HYDROXAMIC ACIDS

(a) *Formation of Hydroxamic Acids*

Pure samples of esters of simple saturated fatty acids containing from one to ten carbon atoms were converted to the corresponding hydroxamic acids by a method similar to that previously described (Thompson 1950). The reaction mixture contained 2 ml. ester or mixture of esters in ethanol (total

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concentration $4 \times 10^{-2}M$), 30 ml. ether, and 1.0 ml. hydroxylamine solution (prepared from equal volumes of 5 per cent. hydroxylamine hydrochloride and 12.5 per cent. sodium hydroxide in methanol, the sodium chloride being removed by filtration).

The mixture was allowed to stand at 25°C. for 30 minutes and 0.08 ml. glacial acetic acid added. The sodium acetate was filtered off and the ether removed under reduced pressure.

(b) Description of Chromatographic Technique

Portion of the solution of hydroxamic acids (containing about 10^{-5} mole of total hydroxamic acids) was applied as a spot about 1.5 in. from the edge of a filter paper sheet (Whatman No. 1). The paper was formed into a cylinder and the chromatographic separation carried out at 20°C. for 16-20 hours as described by Williams and Kirby (1948). In this time the front had travelled 10-20 in., the rate being fastest with benzene as mobile solvent and slowest with octyl alcohol.

The non-aqueous solvent was shaken with water and acid to obtain a mixture of two phases. The acid was used to repress ionization and prevent tailing (see Lugg and Overell 1948 and Section III (b) below). After allowing the solvent mixture to come to equilibrium at 20° C., the aqueous phase was placed in the bottom of the tank outside the dish carrying the paper and the mobile water-poor phase, and, initially, no other means of humidifying the air of the tank was used. In later experiments the air was humidified by lining the tank with paper soaked with the aqueous phase.

Owing to the instability of the hydroxamic acids, the papers were dried at room temperature. The spots were revealed by spraying the paper either with ferric chloride solution (10 per cent.) when the purple spots appeared against a yellow background, or with ferric perchlorate solution (containing approximately 2.7 g. iron and 250 g. perchloric acid per litre) when the background was white. With the perchlorate spray the colours began to fade after a few hours, except the colour from formhydroxamic acid, which faded noticeably in a few minutes. Using the ferric chloride spray the colour was stable for one to two weeks. In either case, a permanent photographic record was obtained (on plain process film using a medium yellow filter).

Table 1 shows the R_F values of a number of unbranched hydroxamic acids for various acidified solvent mixtures. Photographs of some of the corresponding chromatograms are given in Plate 1, Figures 1-6.

III. FACTORS AFFECTING THE CHROMATOGRAPHIC SEPARATION

(a) Composition of Mobile Phase

Separation of hydroxamic acids containing one to five carbon atoms was possible with butanol (Plate 1, Fig. 1) or amyl alcohol (Plate 1, Fig. 2), as was also shown by Fink and Fink (1949). With octyl alcohol (Plate 1, Fig. 3), the separation of hydroxamic acids containing from two to six carbon atoms was achieved. The useful range of separation for benzene-acetic acid (Plate 1,

TABLE I
R_F VALUES OF UNBRANCHED HYDROXAMIC ACIDS

Non-aqueous Solvent	Butanol 40 ml.	Amyl Alcohol 40 ml.		Amyl Alcohol 75 ml.	Amyl Alcohol 100 ml.	Octyl Alcohol 75 ml.	Octyl Alcohol 50 ml.	Octyl Alcohol 50 ml.	Benzyl Alcohol 35 ml.	Benzene 75 ml.	Benzene 100 ml.	Benzene-Octyl Alcohol 37.5 ml.-50 g.				
Acid	Acetic 10 ml. (glacial)	Acetic 10 ml. (glacial)	Acetic (glacial)	Formic 25 ml. (36N)	Sulphuric 0.3 ml. (36N)	Formic 25 ml.	Oxalic 0.5 g. (hydrate)	Sulphuric (36N)	Acetic 20 ml. (glacial)	Formic 75 ml.	Acetic 75 ml.	Formic 100 ml.				
Water	50 ml.	50 ml.	50 ml.	75 ml.	100 ml.	75 ml.	50 ml.	50 ml.	45 ml.	75 ml.	100 ml.	150 ml.				
Experiment number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Figure number	1		2				3					4		5	6	
Formhydroxamic Acid C1	0.42		0.26				0.08					0	0		0.03	0.52
Acethydroxamic Acid C2	0.50	[0.53]	0.34	[0.35]	0.30	[0.30]	0.11	[0.14]	[0.10]	[0.11]	[0.49]	0	0.01	[0]	0.05	0.65
Propionhydroxamic Acid C3	0.62	[0.66]	0.51	[0.55]	0.50	[0.51]	0.25	[0.30]	[0.23]	[0.25]	[0.62]	0.01	0.05	[0.05]	0.14	0.77
Butyrohydroxamic Acid C4	0.72	[0.77]	0.67	[0.71]	0.68	[0.69]	0.45	[0.49]	[0.43]	[0.44]	[0.72]	0.04	0.13	[0.13]	0.29	
Valerohydroxamic Acid C5	0.79	[0.84]	0.78	[0.82]	0.81	[0.82]	0.67	[0.69]	[0.69]	[0.69]	[0.79]	0.11	0.32	[0.30]	0.53	
Hexanohydroxamic Acid C6	0.84	[0.91]	0.86	[0.86]	0.83	[0.85]	0.81	[0.80]	[0.81]	[0.82]	[0.89]	0.26	0.61	[0.55]	0.73	
Heptanohydroxamic Acid C7	0.86	[0.94]	0.89	[0.88]	0.85	[0.85]	0.88		[0.90]	[0.86]	[0.91]	0.51	0.87	[0.76]	0.80	
Octanohydroxamic Acid C8	0.88		0.89				0.90					0.77	0.95	[0.90]	0.89	
Nonanohydroxamic Acid C9	0.89	[0.92]	0.89	[0.90]	0.90	[0.86]	0.90		[0.94]	[0.85]	[0.91]	0.83	0.97	[0.93]	0.93	
Decanohydroxamic Acid C10	0.90		0.90				0.92					0.92	0.97	[0.94]	0.93	

R_F values enclosed in square brackets were obtained when there was no humidification of the system other than the aqueous phase on the bottom of the tank. Otherwise the tank was humidified by lining the tank with paper soaked with the aqueous phase. The lines indicate the limits of usefulness of the separation.

Fig. 5) was three to eight and usually nine carbon atoms. With benzene-formic acid (Plate 1, Fig. 4) the useful range was from four to nine and sometimes ten carbon atoms. Good separations of two to seven carbon atoms were obtained with a mixture of benzene, octyl alcohol, and formic acid (Plate 1, Fig. 6).

Separations from two to four carbon atoms were obtained with benzyl alcohol, but the spots were rather indiscrete. Light petroleum and xylene were of no value. Phenol gave comparatively high R_F values and had a tendency to distort the spots. Although of no use for separating the higher members of the series, it gave a useful separation of the one and two carbon atom hydroxamic acids, as previously found by Fink and Fink (1949). These workers found phenol especially useful for separating hydroxamic acids derived from dicarboxylic acids, which, in general, have lower R_F values.

(b) Acid

The acid was added to the solvents with the object of suppressing the ionization of the hydroxamic acids and thus preventing "tailing" (Lugg and Overell 1948). This is a desirable precaution, although satisfactory chromatograms have sometimes been obtained, both by Fink and Fink (1949) and the author, after applying the hydroxamates in approximately neutral solution and without addition of acid. Fink and Fink (1949) did not use additional acid, and their R_F values indicate that the un-ionized hydroxamic acids travelled forward on the paper. The author has omitted the addition of acid and obtained satisfactory chromatograms with butanol but never with benzene.

It is evident that the ionization of the weak hydroxamic acids is suppressed at a comparatively low hydrogen ion concentration, which may be already attained in the approximately neutral spot applied to the paper. However, it is unwise to rely on this, as the effect may be due to a very slight excess of the acid used for neutralization and it is better to ensure an adequate excess by adding acid to the solvents.

Provided the composition of the mobile phase was not markedly altered by the addition of acid, i.e. if only a small quantity was used, the R_F values were practically independent of the particular acid used. On the other hand, by marked changes in the proportions of acetic acid and benzene, resulting in marked alteration of the composition of the mobile phase, considerable changes in the R_F values were obtained (Table 2).

The author has used a large variety of acids. The choice has not been limited, as in the separation of carboxylic acids by Lugg and Overell (1948), to using a volatile acid since the test used for the detection of hydroxamic acids is specific for these substances in the presence of other acids.

(c) Humidification

Hanes and Isherwood (1949) have adopted special measures to ensure that the paper is allowed to become approximately saturated with respect to the aqueous phase. In this laboratory, it has been found that when the non-aqueous solvent was benzene, which dissolves very little water, the amount of

water vapour in the system has a very important effect on the forward movement of hydroxamic acid spots. If no additional means were adopted to humidify the air in the tank when benzene-formic acid was used, the chromatogram on development frequently showed a continuous streak starting from the point of application of the spot and fading off in the direction of solvent flow without differentiation into individual spots (Plate 2, Fig. 7). This "streaking" is easily distinguished from the "tailing" occurring in the absence of acid in which the spots fade off in the opposite direction.

This phenomenon was always encountered when large papers or a number of concentric papers were used under these conditions. If the air in the tank was humidified by lining the sides of the tank with filter paper soaked in the aqueous phase, this trouble could be avoided (Experiment 12, Plate 1, Fig. 4). The effect of this treatment, designed to assist equilibration between paper, gas phase, and liquid aqueous phase, was to reduce the R_F values slightly.

TABLE 2
EFFECT OF ALTERING THE PROPORTIONS OF BENZENE AND ACETIC ACID
ON THE R_F VALUES OF UNBRANCHED HYDROXAMIC ACIDS

Solvent Proportions			R_F Values of Hydroxamic Acids with the Number of Carbon Atoms shown									
Benzene (ml.)	Acetic Acid (glacial) (ml.)	Water (ml.)	2	3	4	5	6	7	8	9	10	
100	100	100	0.03	0.09	0.21	0.46	0.82	0.96		0.98		
100	75	100	0	0.05	0.13	0.30	0.55	0.76	0.90	0.93	0.94	
100	50	100	0.02	0.05	0.12	0.30	0.55	0.78		0.93		
100	20	100	0	0.03	0.03	0.17	0.40	0.69		0.93		

(d) *Impurities in the Filter Paper*

With benzene and acetic acid a peculiar effect was noticed. Before developing the chromatogram, wedge-shaped, faintly yellow areas were observed on the paper which, on spraying with iron solution, appeared as pale purple areas lying immediately in front of each hydroxamic acid spot in the direction of solvent flow (Plate 2, Fig. 8). This "shadow" effect was most intense in colour with the hydroxamic acid spot of highest R_F value in the sample and most elongated with valerate and other members of the series located near the middle of the paper.

By extracting the paper before use with 50 per cent. acetic acid, washing with water, pressing it between filter paper sheets and drying, the "shadow" effect was almost entirely eliminated.

Two samples of filter paper (Whatman) had an iron content of 19 and 6.3 p.p.m. A polarographic analysis of an extract of the second sample of filter paper showed the following other metals to be present: copper (3.0 p.p.m.), lead (11.4 p.p.m.), zinc (4.2 p.p.m.), nickel (1.4 p.p.m.). The chromium concentration was too low to be analysed polarographically and cobalt and cadmium were not detected.

The "shadowing" was reintroduced by soaking the acetic-extracted filter paper in 10^{-4} molar ferric chloride solution, pressing between filter paper sheets, and drying. With ferric chloride solution of 10^{-2} molar concentration the effect was also observed, but it was less typical, with a large area of shadow compared with the spot area. Moreover, the shadow in this case was purple in colour even before spraying with iron solution. Of the other metals present in the filter paper, only copper and nickel form coloured hydroxamate complexes. Neither of these metals reproduced "shadowing" when introduced into acetic-extracted paper in the same way from 10^{-4} molar solutions. The effect is explained by the ferric ions in the paper forming complexes with the hydroxamic acids with somewhat higher partition coefficients between benzene and water than the free hydroxamic acids and thus preceding them along the paper.

It is of interest that Hanes and Isherwood (1949) have described a somewhat similar shadowing in the separation of phosphoric esters on paper. In this case the shadows lagged behind the main spot. They were attributed to the presence of heavy metals in the paper as the interference could be removed either by extraction of the paper with aqueous alcoholic 8-hydroxyquinoline or by exposing the paper to a small amount of hydrogen sulphide gas before running the chromatogram. It was not removed by extraction with hydrochloric acid. In contrast, the shadowing experienced with the hydroxamic acids could be removed by extraction with hydrochloric acid, but acetic acid was preferred as having less effect on the strength of the paper.

IV. DISCUSSION

To the author's knowledge, the separation of hydroxamic acids containing one to nine carbon atoms represents the largest number of consecutive members of a homologous series (differing only by a $-\text{CH}_2-$ group) which have been separated by the paper chromatographic technique. No particular advantage was obtained by using the two-dimensional chromatographic technique for the separation of the homologous series of hydroxamic acids, and two separate one-dimensional chromatograms, with solvents that separate the lower and higher members of the series respectively, were more convenient.

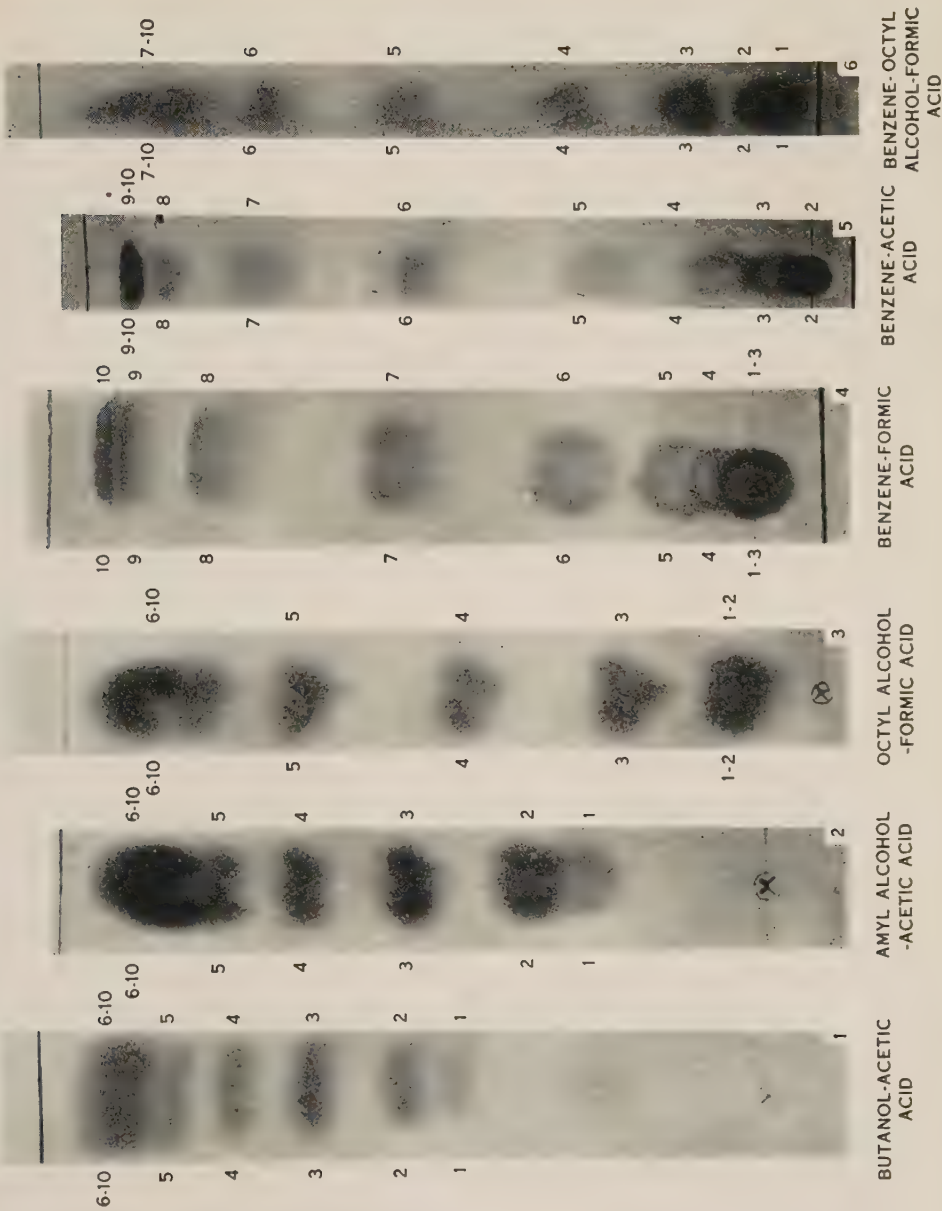
The method described permits the identification of the lower members of a homologous series of any substances that can be converted to hydroxamic acids—esterified acids, acids, alcohols (after oxidation), and aldehydes. The latter react directly with toluenesulphonhydroxamic acid to form hydroxamic acids. Details of these procedures will be given in a subsequent paper describing the use of this technique for identifying apple volatiles.

V. ACKNOWLEDGMENTS

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VI. REFERENCES

- CONSDEN, R., GORDON, A. H., and MARTIN, A. J. P. (1944).—Qualitative analysis of proteins: a partition chromatographic method using paper. *Biochem. J.* **38**: 224-32.
- FINK, K., and FINK, R. M. (1949).—Application of filter paper partition chromatography to the qualitative analysis of volatile and non-volatile organic acids. *Proc. Soc. Exp. Biol.* **70**: 654-6.
- HANES, C. S., and ISHERWOOD, F. A. (1949).—Separation of the phosphoric esters on the filter paper chromatogram. *Nature*. **164**: 1107-12.
- LUGG, J. W. H., and OVERELL, B. T. (1948).—"One" and "two-dimensional" partition chromatographic separations of organic acids on an inert sheet support. *Aust. J. Sci. Res. A* **1**: 98-111.
- THOMPSON, A. R. (1950).—A colorimetric method for the determination of esters. *Aust. J. Sci. Res. A* **3**: 128-35.
- WILLIAMS, R. J., and KIRBY, H. (1948).—Paper chromatography using capillary ascent. *Science*. **107**: 481-3.



Chromatograms illustrating the separation of hydroxamic acids in various solvents. The numbers beside the spots indicate the number of carbon atoms in the hydroxamic acids. The point of application of the solute is indicated at the bottom of the sheet and the solvent front at the top.

SEPARATION OF MONO-HYDROXAMIC ACIDS

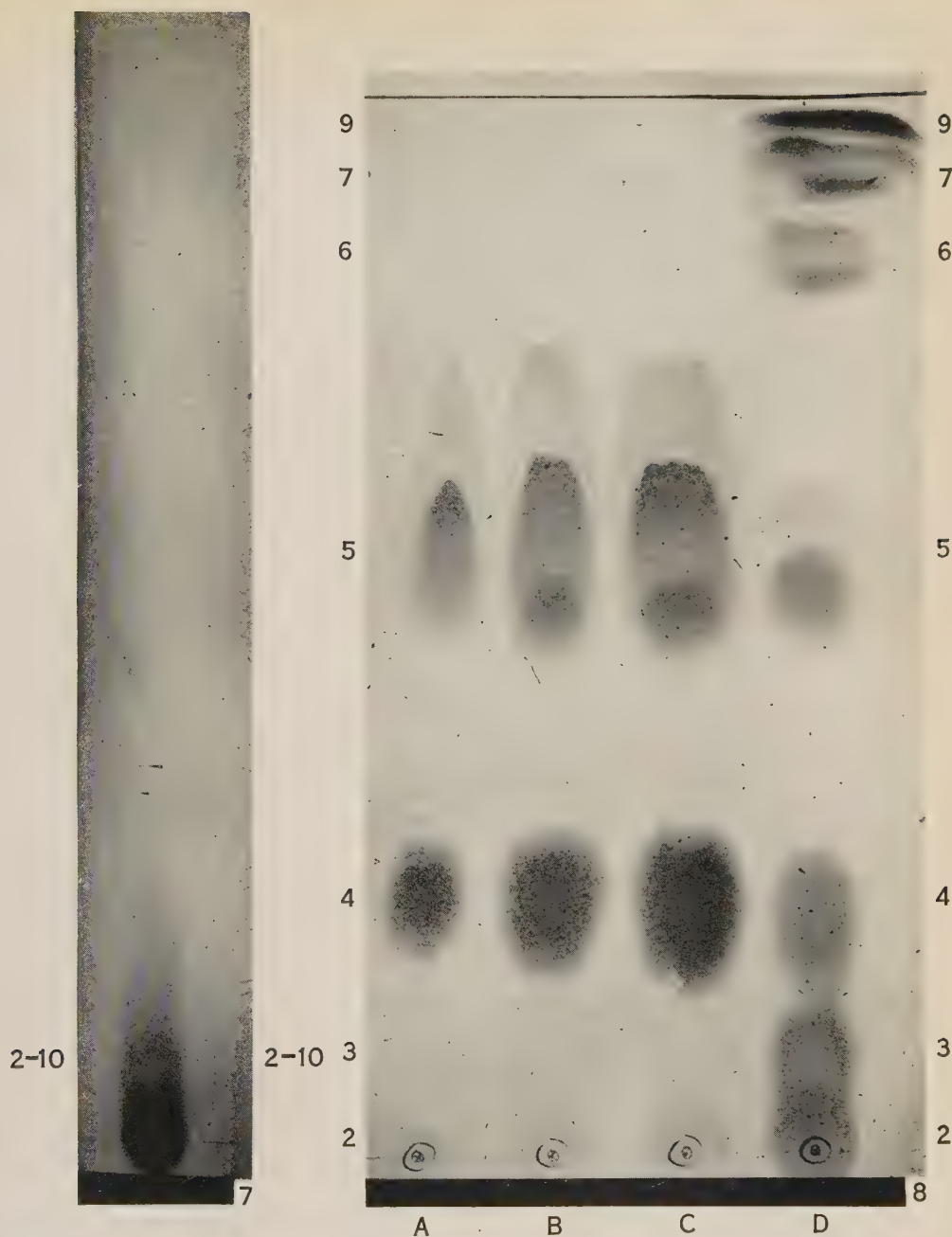


Fig. 7.—Benzene-formic acid. Chromatogram showing “streaking” due to insufficient humidification. Spot contained hydroxamic acids with two to ten carbon atoms.

Fig. 8.—Benzene-acetic acid. Chromatogram showing “shadows” caused by impurities in the filter paper. (The total quantity of hydroxamic acids in samples B, C, and D was twice that in sample A.)

STUDIES ON THE DEPILATORY ACTIVITY OF SODIUM SULPHIDE AND SOME RELATED COMPOUNDS

By J. M. GILLESPIE*

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Summary

The depilatory action of sodium sulphide on sheepskin has been investigated by measuring the pull required to remove wool from skin, the swelling of wool root shafts, and the mechanical strength of wool. The rate of action is augmented by increase in temperature, pH value, and concentration of sulphide in the solution employed up to 1.0M, but is reduced by increase to higher concentrations. The reciprocal action of the hydroxyl and hydrosulphide ions in digesting keratin is explained in Appendix I in terms of the ionization of sulphhydryl groups.

Solutions of 1.0M sodium sulphide, partially oxidized with peroxide and adjusted to pH 12.8, completely loosen wool in 18 hours at 20°C. only if the oxidation-reduction potential is lower than —400 millivolts. Salts of various oxyacids of sulphur are less effective depilatories than sodium sulphide, the activity diminishing with increase in the number of oxygen atoms in the anion and approximately with the heat of formation.

Sodium selenide and sodium telluride possess depilatory activity equal to that of sodium sulphide.

I. INTRODUCTION

For hundreds of years, chemicals have been used in the leather industry to remove hair, wool, and epidermis from skins and hides prior to tanning. Lime, alone or "sharpened" with arsenic sulphide, was used originally, but recently sodium sulphide was found to have pronounced depilatory powers and it is today the most widely used adjunct to lime. Merrill (1925), Marriott (1928), and others have determined the optimal conditions for the un-hairing of hides. The findings of these workers are not directly applicable to the problem of de-wooling sheepskins for, whereas hair is of so little value that in the tannery the usual procedure is to immerse the hides completely in the un-hairing solution, wool is such a valuable product that precautions must be taken to prevent damage by the alkaline solutions used to loosen it from the skin. Consequently, the technique of applying the depilatory as a paste to the flesh side of the skin has been developed to minimize contamination of the wool.

This chemical de-wooling process has received practically no scientific investigation in the past and it is still largely empirical. The viscosity of the paste, the concentration of sodium sulphide, and the time of application vary from place to place and, although the concentration is sometimes varied according to the thickness of the skins, strict control is seldom exercised. Usually,

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too, the wool is removed approximately 24 hours after application of the depilatory to the skins, regardless of the hastening or retarding influences of such factors as temperature and thickness of skin.

If the depilatory has low viscosity, and is therefore easily applied, excess may contaminate the wool at the edge of the skin and weaken or even dissolve it. Contamination also occurs when the wool is pushed off the skin onto the depilatory-contaminated pulling beam. In addition, the calcium compounds in this contaminated wool and on the root ends of the detached wool precipitate calcium soaps during scouring and contribute further to deterioration in the quality of the wool. Consequently, wool obtained by chemical loosening may be characterized by a harsh handle, lack of lustre, and the presence of particles of calcium salts, disintegrated wool, and weakened fibres, which break up and are lost during combing.

It was with the object of defining more accurately the minimum requirements for depilatory activity, with special reference to the influence of physical and chemical factors, that the present investigation was undertaken.

II. METHODS OF INVESTIGATION

(a) Preparation of Reagents

Except when otherwise stated, the reagents used were of analytical reagent quality or better. The sodium salts of hydrogen sulphide, selenide, and telluride were specially prepared.

Sodium sulphide was prepared from the chemically pure salt, hydrogen sulphide being liberated by mixing it with hot hydrochloric acid, washed by bubbling through water, and absorbed in pure sodium hydroxide. This solution of sodium hydrosulphide was stored in sealed paraffin wax-lined bottles and diluted with water and adjusted to the required pH with sodium hydroxide immediately before use. Although, at the pH values used in these investigations, the material was actually a mixture of sodium sulphide and hydrosulphide, for convenience it is referred to as sodium sulphide and its properties are accurately defined by the pH and the molarity in terms of the hydrogen sulphide content.

Sodium selenide and sodium telluride were prepared immediately before use by direct combination of the elements at an elevated temperature in the absence of oxygen (Vallance, Twiss, and Russell 1931). The exact stoichiometric weights of the elements were used to avoid the formation of polycompounds. Sodium selenide was also prepared by reduction of sodium selenate with carbon, and by combination of the elements in the vapour phase at low pressure.

The kieselguhr and kaolin employed were finely ground commercial mineral specimens.

(b) Preparation of Depilatories

The chemical substances used in the depilatories were dissolved in distilled water. The actual content of active reducing material was determined by standard iodometric procedures (Hall 1936). Unless otherwise stated, the

solutions were adjusted to a suitable physical state with kieselguhr. Before use, the pH of the depilatory was adjusted to the required value by the addition of hydrochloric acid or sodium hydroxide. For pH measurements, a Leeds and Northrup valve potentiometer was used in combination with a Cambridge high alkalinity glass electrode.

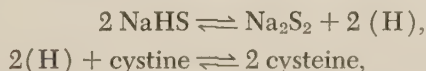
(c) *Measurement of Wool Loosening Activity*

The method of measuring wool loosening was that due to Lennox (1945). From the central area of a fresh sheep skin, pieces 5×5 cm. were cut, and blood and dirt removed by thorough rinsing in running water. In most experiments the skin pieces were then immersed for 30 min. in an alkaline buffer of the same pH as the depilatory to be applied. Sodium carbonate (0.2M) or 0.2M trisodium phosphate was used for this purpose.

A measured amount of the depilatory was applied to the flesh side of the skin, covered with a glass plate to restrict evaporation and oxidation, and the whole then placed in a moist chamber at 20°C. The progress of wool loosening was followed by the determination, at suitable time intervals, of the depilation load. A depilation load of less than 1 was considered to indicate complete loosening of the wool.

(d) *Measurement of Eh*

Any chemical process that depends for its efficiency on reduction must be influenced by the reducing intensity of the system. The normal measure of this, particularly in biological systems, is the oxidation-reduction potential, but this only applies if the system is reversible. In the system now under study there are the reversible reactions:



and the irreversible oxidation of sulphide to sulphite or thiosulphate.

The irreversible nature of the latter reaction and the ease with which the platinum electrodes are poisoned by systems containing sulphydryl compounds render Eh measurements difficult and of doubtful accuracy. However, some measurements were made using a valve potentiometer and platinum electrodes similar to those described by Gillespie (1946). The electrodes were cleaned in boiling concentrated nitric acid after each reading, a mean value from four electrodes being obtained for each Eh. The potentials were converted to the H_2 standard and the results given later are presented in this form.

(e) *Fibre Strength Measurements*

Histological observation has shown that most de-wooling chemicals act by reducing the strength of the keratinous region of the wool root, and consequently the mode of action of depilatories can be investigated by determining their effect on wool fibres.

The single fibre breaking-strength apparatus described by Fookes (1946) was used in these investigations. The technique is open to the criticism that

no provision was made for constant loading rates or for maintaining a humidified atmosphere. These were overcome by care in operation and the inclusion of samples of wool of known strength at frequent intervals during testing. Observations on these revealed any change in testing conditions. With these precautions, satisfactory comparative figures were obtained.

The chemicals under test were dissolved in air-free distilled water, sometimes with the addition of the wetting agent, cetyl trimethylammonium bromide. The wool used was merino 60-64's cleaned by extraction with solvents in the following order: high aromatic light petroleum, acetone, water, acetone. About 30 kg. of wool was thus treated and after careful picking to remove foreign matter, it was thoroughly randomized. It had a normal content of cystine and possessed no detectable sulphydryl groups.

(f) *Wool Root Studies*

The wool fibres with roots attached were pulled from a sheep each day. The roots, consisting of root bulb with about 5 mm. of attached shaft, were placed in the cavity of a well slide, a few drops of the reagent under test was added, and the preparation covered with a slip and placed on the stage of a dissecting microscope. Swelling was detected and semi-quantitatively measured with an eyepiece micrometer. There was usually a lag period before swelling commenced, followed by a period of rapid expansion lasting about 30 sec., and finally a much longer period during which the rate of swelling became progressively slower. The recorded time of swelling was that required for the completion of the lag and rapid phases.

III. EXPERIMENTAL RESULTS

(a) *Influence of Various Conditions on the Depilatory Activity of Sodium Sulphide*

(i) *pH*.—Four procedures were used to determine the pH at which optimum wool loosening occurs. In the first, duplicate skin pieces were soaked in buffers over the range pH 9 to 13 and then were treated with sufficient depilatory, containing 1.0M sodium sulphide at the same pH as the buffer used, to give a standard weight per unit area of skin. Depilation loads were measured at $\frac{1}{2}$, 1, 2, 4, and 18 hours at 20°C. The results at 18 hours are plotted in Figure 1A. Variation in the thickness of skin pieces caused errors in the above determinations. The second method eliminated these errors. A whole sheepskin was treated with a 1.0M sodium sulphide depilatory, and after 24 hours, depilation loads and surface pH values were determined at a number of positions on the skin. The results of this experiment are given in Figure 1B.

The third method demonstrated a relationship between the pH of solutions of sodium sulphide and the rate of swelling of the shafts of wool roots immersed therein. This gave an indication of the conditions required for initial weakening of the wool root shaft in the follicle. The results are given in Figure 2. In the fourth method, the relation between pH of a sodium sulphide solution

and its effect on the breaking strength of wool fibres was determined, the concentration being maintained constant by the use of a very large ratio of solution to wool. These results are shown in Figure 3.

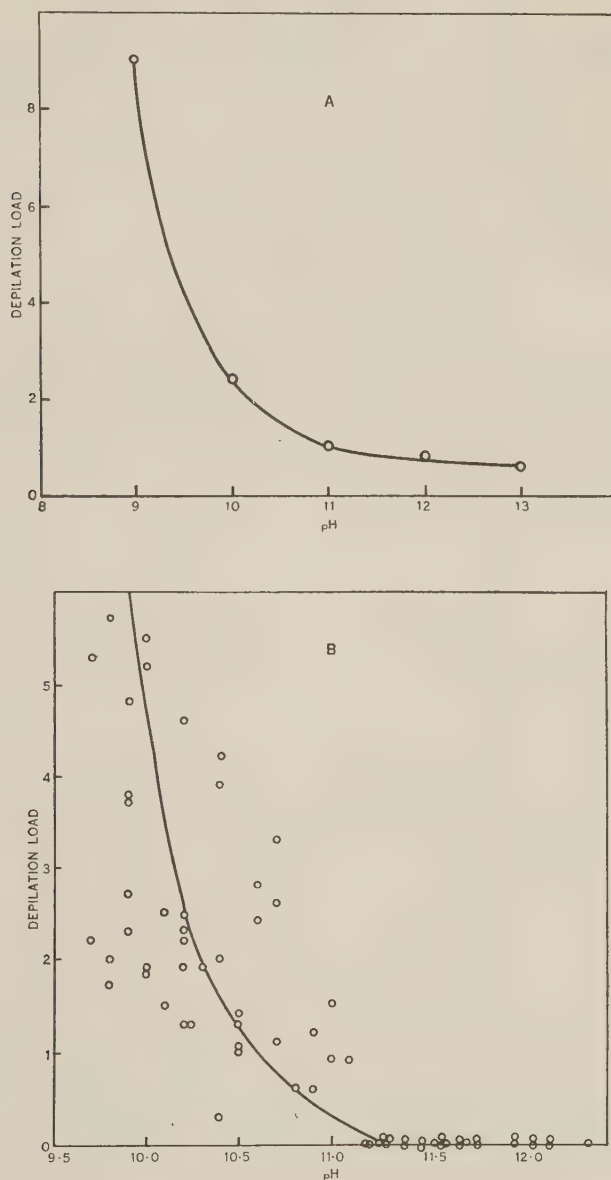


Fig. 1.—A. Relationship between pH of 1.0M sodium sulphide solutions and depilation load.

B. Relationship between pH of 1.0M sodium sulphide solutions and depilation load. (Determined by measuring local surface pH values and corresponding depilation loads.)

It is interesting to observe the profound influence of pH on the extent of wool loosening and wool damage. The approximate effective values of pH are tabulated in Table 1. An explanation of the differences in pH value will be suggested later.

TABLE 1
MINIMUM pH VALUES AT WHICH 1.0M SODIUM SULPHIDE EXERTED A SIGNIFICANT EFFECT ON WOOL ROOTS AND WOOL FIBRES

Reaction	pH at which Reaction is Extensive
Wool root shaft swelling	9.5
Wool loosening	11.0
Reduction in strength of wool fibres	11.5

(ii) *Concentration of Sodium Sulphide*.—Two methods were used to determine the effect of concentration of sodium sulphide on the depilatory action. In the first method, several skin pieces were soaked in buffers at five pH values

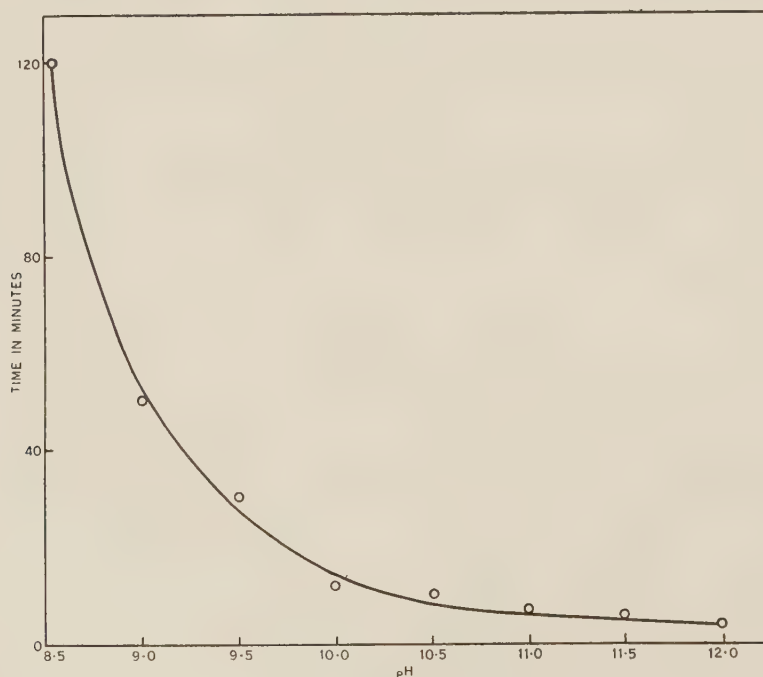


Fig. 2.—Influence of pH on the time taken for 1.0M sodium sulphide to cause visible swelling of the wool root shaft.

and then treated with depilatories at the same pH values but containing concentrations of sodium sulphide covering the range of 0.01 to 1.0M. In Figure 4 the depilation loads for such a series of skin pieces cut from one skin are plotted against the logarithms of the sodium sulphide concentration. In some experiments, the concentration of sodium sulphide was increased beyond the usual

1.0M. It was then observed that instead of increasing further, the activity actually decreased (Fig. 5). These results could be reproduced by adding sodium chloride to a 1.0M solution of sodium sulphide. It therefore seems probable that the osmotic effect of high salt concentrations opposed the depilatory effect of the sulphide. In the second method, reported in Figure 6, the relationship between sodium sulphide concentration and the breaking strength of wool fibres at six different pH values was determined.

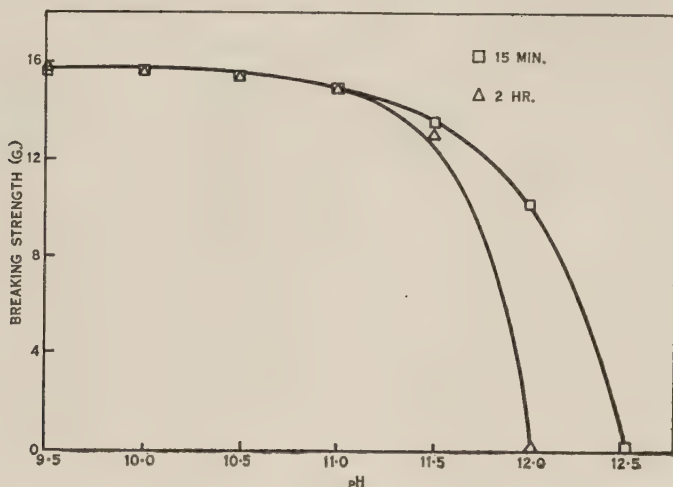


Fig. 3.—Relationship between the pH of 1.0M sodium sulphide solutions and their ability to lower the breaking strength of the wool.

It is apparent that there is an interrelation between pH and the concentration of sodium sulphide solution as measured by either wool loosening activity or the extent of wool damage. The most noteworthy point is the effectiveness of very weak solutions at the higher pH values. Thus 0.025M sodium sulphide at pH 13 is as effective as 0.25M solution at pH 12 and 0.75M solution at pH 11.5, whilst even in 1.0M concentration, solutions at pH 11.0 and 10.5 could not reduce the breaking strength to zero in the time allowed. They did so, however, over longer periods.

(iii) *Oxidation-Reduction Potential*.—A series of nine aliquots of 1.0M sodium sulphide were partially oxidized by nine hydrogen peroxide solutions having graded concentrations such that the most concentrated was just sufficient to completely oxidize the sulphide. A tenth aliquot of sodium sulphide was used in the completely unoxidized state. The pH was maintained at 12.5 during the oxidation by the occasional addition of NaOH.

The ten solutions were thickened with kieselguhr and the pH adjusted to 12.8. Oxidation-reduction potential measurements were made on a portion of each and the remainder was applied in duplicate to skin pieces. The depilation load was measured after 18 hours at 20°C. Figure 7 shows that wool loosening became detectable at potentials more negative than -300 millivolts and

complete loosening was accomplished at potentials more negative than -400 millivolts.

(iv) *Temperature and Time*.—As would be expected, depilation load is also a function of temperature and time of action of the depilatory. The curves in Figure 8 were obtained when skin pieces that had been preheated to the temperature of the experiment were treated with $0.5M$ sodium sulphide depilatory adjusted to pH 12.8 and preheated to the same temperature as the skin to which it was applied. The temperature was maintained constant during the experiment and depilation loads were measured at suitable intervals of time. The higher the temperature, the more rapidly the depilation load diminished. The upper limit was, of course, set by the low shrinkage temperature of the skin in the presence of $1.0M$ sodium sulphide, which is $53^{\circ}C$. (Lennox, unpublished data).

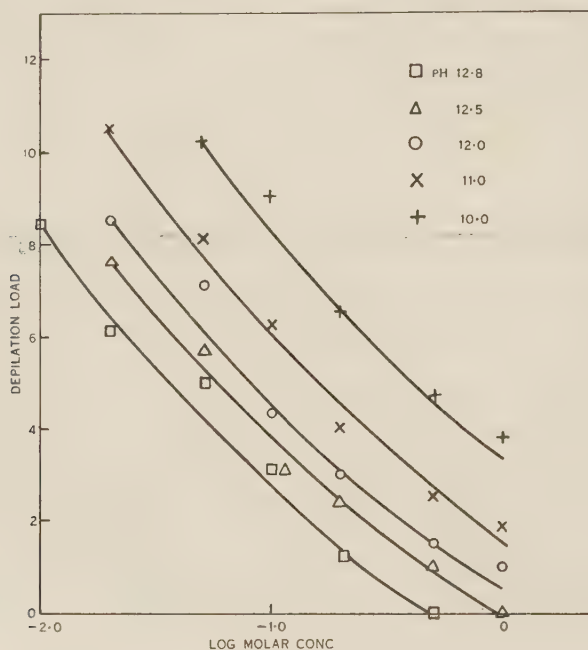


Fig. 4.—Relationship between concentration of sodium sulphide and depilation load after 18 hours at various pH values at $20^{\circ}C$.

(b) *Depilatory Activity of Various Inorganic Compounds Containing Sulphur in the Anion*

Depilatories at pH 12.8 containing the usual amount of kieselguhr but with the sodium sulphide replaced by sodium salts containing sulphite, sulphate, thiosulphate, dithionite, dithionate, or trithionate ions, respectively, were tested in the usual way on buffered skin pieces. The results are given in Table 2, together with the heats of formation of the acids from which the salts were

derived (Hodgman and Holmes 1945). It will be noted that, when arranged in order of increasing heat of formation, which is related to reducing capacity,

TABLE 2
DEPILATORY ACTIVITY OF SOME COMPOUNDS CONTAINING SULPHUR IN THE ANION,
TESTED AT 1.0M CONCENTRATION AND pH 12.8

Compound	Formula	Depilation Load After 18 hr.	Heat of Formation of the Corresponding Acid (kcal.)
Sodium hydroxide control	NaOH	36	—
Sodium dithionate	Na ₂ S ₂ O ₆	33	274.31
Sodium tetrathionate	Na ₂ S ₄ O ₆	33	262.37
Sodium trithionate	Na ₂ S ₃ O ₆	33	261.89
Sodium sulphate	Na ₂ SO ₄	34	210.28 (dil.)
Sodium dithionite	Na ₂ S ₂ O ₄	8	166.1 (dil. sol.)
Sodium sulphite	Na ₂ SO ₃	6	145.09 (300)
Sodium thiosulphate	Na ₂ S ₂ O ₃	6	138.6 (1,500)
Sodium sulphide	Na ₂ S	1	9.56 (liquid)

a series is obtained within each group of compounds resembling that obtained by arranging them in order of increasing depilation load.

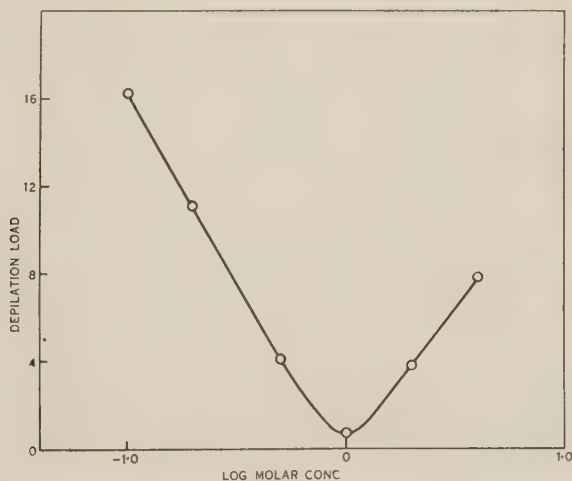


Fig. 5.—Relationship between concentration of sodium sulphide and depilation load showing the inhibitory effect of high concentrations. The samples were incubated for 18 hours at 20°C. before measuring the depilation load; pH was held at 12.5.

Sodium polysulphides up to Na₂S₄ were also prepared and tested and, in general, they showed a decrease in depilatory activity with increase in S content. However, in some experiments it was observed that a mixture of 0.9M sodium sulphide and 0.1M Na₂S₂ showed greater depilatory activity than pure sodium sulphide.

The Australian fellmongering industry relies on an impure commercial grade of sodium sulphide for preparing depilatories. This is usually a fused product containing as impurities polysulphides, carbonates, sulphites, thiosulphates, and sulphates, Al and Si in appreciable amounts, and traces of B, Cu, Ca, and Ga. When depilatories of identical pH, ionic strength, and SH con-

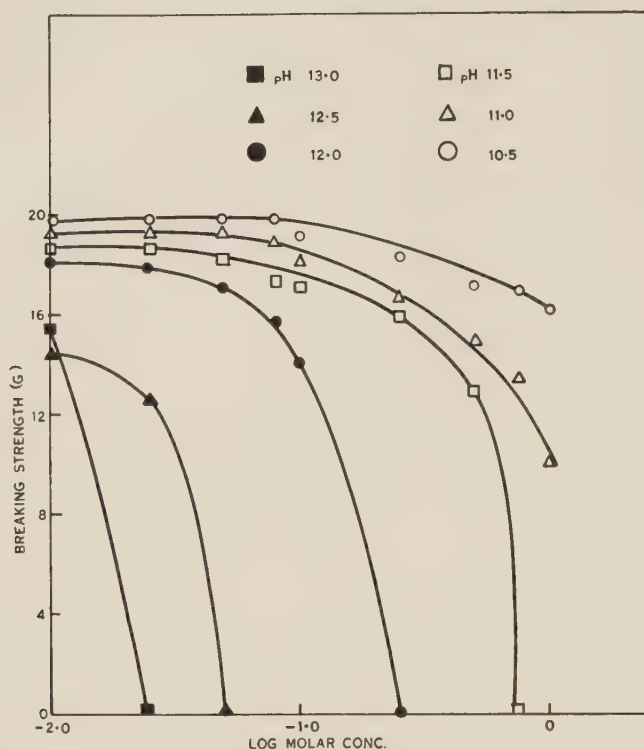


Fig. 6.—Influence of sodium sulphide concentration at various pH values on the tensile strength of wool after 30 min. at 20°C.

tent were tested side by side, one prepared from pure sodium sulphide and the other from crude sodium sulphide, the latter had the greater activity. A typical set of results is shown in Table 3. The effect was most noticeable with

TABLE 3
THE GREATER DEPILATORY ACTIVITY OF IMPURE THAN PURE SODIUM SULPHIDE AT 20°C.

Depilatory	Depilation Load			
	0 hr.	½ hr.	2 hr.	18 hr.
Sodium sulphide 1.0M	58.1	34.3	8.5	0.5
Sodium sulphide 1.0M (crude)	57.5	1.0	0	0

thick skins that were hard to depilate, presumably owing to their high fat content and thick collagen layer.

By fractional crystallization the active fraction was concentrated in the dark brown mother liquor, and the addition of a small quantity of this concentrate to pure sodium sulphide exerted a marked activating effect. The mother liquor was rich in polysulphides but its activating effect could not be reproduced by synthetic polysulphides prepared by dissolving sulphur in sodium sulphide.

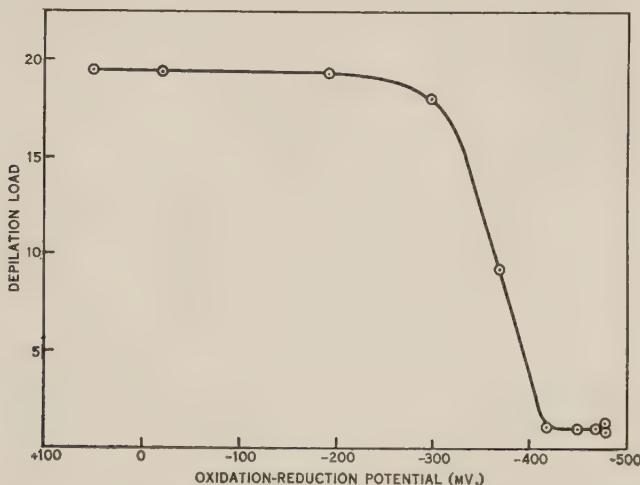


Fig. 7.—Influence of oxidation-reduction potential on the rate of wool loosening by oxidized 1.0M sodium sulphide solutions at pH 12.8 after 18 hours at 20°C.

(c) Depilatory Activity of some Compounds of Selenium and Tellurium

In an attempt to determine what impurities in commercial sodium sulphide are responsible for its high depilatory activity (because of their own rapid depilatory action or by catalysis of the action of sodium sulphide) an examination was made of some of the impurities that are most likely to promote activity. These were considered to be other sulphur compounds and substances containing the related elements, selenium and tellurium. When investigating this latter possibility, it was observed that the alkali and alkaline earth metal salts of hydrogen selenide and telluride in suitable concentration and at pH's greater than 11 were very active as depilatories. Some of the results are shown in Table 4.

Under the conditions of the experiment, sodium selenide and telluride appear to have much the same pH threshold as sulphide and possess approximately the same depilatory activity as sulphide when compared on a molar basis.

IV. DISCUSSION

Some divergence can be observed between the minimum pH values for the action of sodium sulphide as determined by various methods. Thus, rapid swelling of wool root shafts occurred at a lower pH than depilation and this, in turn, was lower than the pH associated with measurable reduction in the

strength of wool fibres. A possible reason is that swelling of the wool root is only an early stage in the weakening of the root that is necessary for depilation, whilst the more drastic treatment required for wool fibre damage may be

TABLE 4
DEPILATORY ACTIVITY OF 1.0M SOLUTIONS OF SODIUM SULPHIDE AND SODIUM TELLURIDE AT pH 13, AND OF SODIUM SELENIDE AT VARIOUS pH VALUES, AT 20°C.

Time (hr.)	Depilation Load					
	Na ₂ S at pH 13	Na ₂ Se at the Following pH Values:				Na ₂ Te at pH 13
		13	12.5	12	11.5	
0	32	29	35	35	34	30
½	34	23	39	35	34	33
1	17	16	19	23	24	20
2	9	8	7	9	10	8
18	0	0	0	2	3	0

due to the resistant nature of this material as compared with the newly formed keratin of the wool root shaft. Below pH 10 depilation is difficult to achieve, even with concentrated solutions of reducing agents, and this is probably associated with a corresponding difficulty in completely reducing -S-S- bonds below this pH value (Patterson *et al.* 1941).

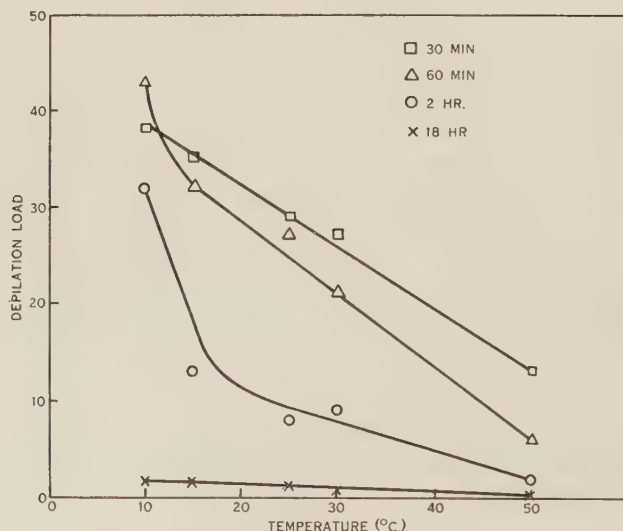


Fig. 8.—Influence of temperature on the rate of wool loosening by 1.0M sodium sulphide at pH 12.8.

Inhibition of depilation by high concentrations of sodium sulphide or by sodium sulphide plus sodium chloride was observed on many occasions. The exact cause is probably not simple, but two possibilities suggest themselves.

The first, which lacks experimental evidence, is that, by withdrawing water from the skin tissues, penetration of the depilatory chemicals is retarded. This is in keeping with the observation that depilation is impossible to achieve with dry, or partly dry, skins. The second suggestion is that sodium salts interfere with the attack on keratin. Vago (1937) has stated that an essential part of keratin lysis is reduction of $-S-S-$ bonds and then splitting of the keratin bundles by absorption of water. It has been observed that high concentrations of salts inhibit the swelling of wool root shafts in dilute sodium sulphide solutions and the second explanation therefore seems to be the more likely.

From the experimental data available it is impossible to state an optimal temperature for depilation of sheepskins, as the rate of depilation increases with rise in temperature until the skin shrinkage temperature is reached. It would be expected that increasing the temperature would accelerate diffusion of chemicals through the skin, partly of its own accord and partly by increasing the rate of hydrolysis of fatty layers that hinder penetration. The reaction of sulphide and hydroxyl ions with the wool root shafts would also be accelerated. There may be a temperature at which there is considerable keratin lysis but little damage to other skin proteins, but this has yet to be revealed. Similarly it is impossible to specify at this stage any particular concentration and pH value of sodium sulphide for effective depilation, for the one is dependent on the other, as shown in Appendix I.

TABLE 5

INFLUENCE OF CONCENTRATION AND TIME ON THE pH REACHED IN THE CENTRE LAYERS OF SKIN PIECES IMMERSSED IN SODIUM CARBONATE SOLUTIONS AT 20°C.

Molar Concentration	pH of Solution	pH in Centre Layer of Skin			
		15 Min.	30 Min.	60 Min.	120 Min.
0.1	11.5	—	9.6	10.0	10.3
0.2	11.8	—	9.9	10.1	10.5
0.5	12.2	—	10.0	10.5	11.3
1.0	12.3	9.8	9.9	10.7	11.7

Since depilatories are not applied directly to the wool root shaft but to the flesh side of the skin, the active components OH^- and SH^- must pass through the skin before reaching their site of action. No appreciable reaction takes place between the SH^- and the skin proteins, and a high level of SH^- is found within a few minutes at the epidermal level. However, the skin proteins react with appreciable quantities of OH^- and there is always a marked pH gradient from the flesh side to the wool side. In Table 5 some figures for the penetration of OH^- are given. The pH values were measured by inserting a high alkalinity glass electrode into a cavity formed by slitting the skin, and were confirmed by using indicators.

It is evident that the depilatory not only has to provide OH^- for the reaction with keratin but also has to bring the pH of the skin proteins up to 10-11. In many experiments it has been found that sodium sulphide solutions alone (1-1.5M) do not provide sufficient OH^- , although there is a great excess

of SH^- . It is now clear why lime has proved such a useful thickener for sodium sulphide depilatories, for it provides an adequate reserve of OH^- which at no time can attain a sufficiently high concentration to damage the pelt.

The use of lime has many disadvantages but alternatives are difficult to suggest. The provision in the skin of an initial reserve of OH^- by immersing it in a solution of Na_2CO_3 provides one successful alternative and a second is to be found in the provision in the depilatory itself of extra OH^- by the controlled addition of NaOH .

On theoretical grounds it was anticipated that Na_2Se and Na_2Te would be active depilatories and that there would be an increasing activity up the series Na_2S , Na_2Se , and Na_2Te as evidenced by the relative ease of oxidation of these compounds. Failure to detect a corresponding increase in wool loosening activity might be due to partial oxidation during preparation and application of the depilatories.

V. ACKNOWLEDGMENTS

Dr. S. J. Leach and Mr. E. F. Woods have provided helpful contributions to the theoretical aspects of Appendix I. The authors are also grateful to Miss C. M. M. Mackinnon and Mr. H. R. Day for their technical assistance.

VI. REFERENCES

- BLACKBURN, S., and LINDLEY, H. (1948).—*J. Soc. Dy. Col. Bradford* **64**: 305.
BOWES, J. H. (1949).—*J. Soc. Leath. Tr. Chem.* **33**: 176.
CUTHBERTSON, W. R., and PHILLIPS, H. (1945).—*Biochem. J.* **39**: 7.
FOOKES, R. A. (1946).—*J. Coun. Sci. Industr. Res. Aust.* **19**: 195.
GHOSH, J. C., RAYCHAUDHURI, S. N., and GANGULI, S. C. (1932).—*J. Indian Chem. Soc.* **9**: 43.
GILLESPIE, J. M. (1946).—*J. Coun. Sci. Industr. Res. Aust.* **19**: 117.
HALL, W. T. (1930).—"Text Book of Quantitative Analysis." (John Wiley & Sons Inc.: New York.)
HODGMAN, C. D., and HOLMES, H. N. (1945).—"Handbook of Chemistry and Physics." 29th Ed. (Chemical Rubber Publishing Co.: Cleveland.)
KÜSTER, F. W., and HEBERLEIN, E. (1905).—*Z. anorg. Chem.* **43**: 53.
LENNOX, F. G. (1945).—*Coun. Sci. Industr. Res. Aust. Bull.* No. 184.
MARRIOTT, R. H. (1928).—*J. Int. Soc. Leath. Chem.* **12**: 216, 281, 343.
MERRILL, H. B. (1925).—*Industr. Engng. Chem.* **17**: 36.
OGSTON, A. G. (1947).—*Physiol. Rev.* **27**: 228.
PATTERSON, W. I., GEIGER, W. B., MIZELL, L. R., and HARRIS, M. (1941).—*J. Res. Nat. Bur. Stand.* **27**: 89.
PREISLER, P. W. (1930).—*J. Biol. Chem.* **87**: 767.
VAGO, G. (1937).—"Stiasny Festschrift." p. 407. (Eduard Roether: Darmstadt.)
VALLANCE, R. H., TWISS, D. F., and RUSSELL, A. R. (1931).—"A Text Book of Inorganic Chemistry." **7** (2). (Charles Griffin & Co. Ltd.: London.)

APPENDIX I

THE RECIPROCAL ACTION OF HYDROXYL AND SULPHYDRYL IONS IN THE
DIGESTION OF KERATIN

By W. G. CREWTER* and J. M. GILLESPIE*

A physicochemical treatment of the reduction of the dithiol linkages of wool by alkali sulphides is made difficult firstly, by the heterogeneous nature of the system, and secondly, by our lack of exact knowledge of the reaction taking place. However, the experiments described in this appendix suggest that, over a limited range of pH values, a simple relationship exists between the pH and sulphide concentration of solutions bringing about a constant degree of wool degradation. It is considered therefore that the theoretical treatment here presented is of use in suggesting further experiments, though it cannot be considered rigorous.

It has been suggested by Preisler (1930) that reactions between thiol compounds do not approach a true equilibrium. However, the experiments on which this opinion was based were carried out at very low pH values and it has been shown by Ghosh, Raychaudhuri, and Ganguli (1932) that, at pH values exceeding 7.0, thiol-dithiol systems behave as redox systems in the absence of oxygen. As the experiments to be described were conducted at pH values exceeding 9.0, it has been assumed that a true equilibrium is approached and that the usual electrode equations can be used to describe the system at equilibrium. In view of the inherent inaccuracies in the experimental method, activity coefficients have been neglected.

The relationship between the limiting values of pH and sulphide concentration necessary for reduction of the tensile strength of wool to zero was determined as follows:

A solution of potassium sulphide, prepared from A.R. potassium hydroxide and washed hydrogen sulphide gas in air-free solution, was suitably diluted with air-free water and aliquots of each dilution were adjusted to various pH values. These solutions were numbered in a random manner prior to setting up the experiments, and aliquots were taken for sulphide estimations by the usual iodometric technique (Hall 1936), the solutions being acidified after mixing with the iodine solution. A 20 ml. portion of the sulphide solution and 0.1 g. of well-mixed solvent-scoured wool were used for each test, and the digestion was carried out in an atmosphere of nitrogen. The pH values of the sulphide-wool mixtures were measured at the conclusion of the experiment, using a Leeds and Northrup potentiometer, a high alkalinity glass electrode, a saturated KCl calomel electrode, and a saturated KCl bridge.

The wool was examined at regular intervals of time and classified according to the degree of retention of its fibre structure, the final observation being made after 20 hr. at 20°C. The degree of softening and disintegration of the wool has been indicated by separate symbols and plotted in Figure 9. It is

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apparent that values of pH and log [sulphide] for solutions bringing about a constant degree of wool damage, for example, that denoted by "X," fall near a straight line with unit negative slope. If the values of pH and log [sulphide] corresponding with an arbitrarily chosen degree of wool damage are plotted for digestion periods of 1, 2, 3, and 20 hr., the points approximate to a straight line in each case, the curve retaining a negative unit slope but being displaced on the coordinates with changes in time (Figure 10).

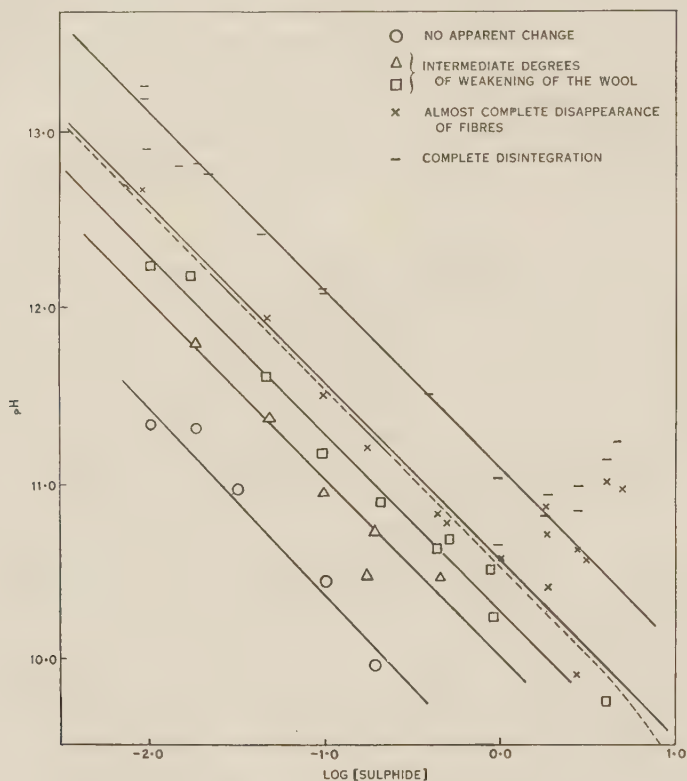


Fig. 9.—Disintegration of wool by potassium sulphide.

Since the results obtained with 3 hr. and 20 hr. digestion periods were practically identical, it is assumed that equilibrium had been reached after the latter period.

At concentrations of K_2S exceeding 1M, the results deviated from a straight line relationship (Fig. 9). This deviation from linearity was accentuated when sodium sulphide was used in place of potassium sulphide, the change then occurring at a concentration of 0.1M (Fig. 11). It was characteristic of such tests that, although the major portion of the wool was completely disintegrated, there remained a rather hard, dense residue. With sodium sulphide also, longer periods were required for the attainment of equilibrium, noticeable changes in the wool taking place for two or three days. It is probably for this reason that sodium sulphide solutions yielded more variable results than

potassium sulphide, when using a digestion period of 20 hr. Addition of sodium chloride to the sodium sulphide solutions retarded the disintegration process still further. In Figure 12, the results obtained with sodium sulphide solutions containing 2M sodium chloride are compared with the line representing almost complete disintegration of wool (\times) obtained with unsupplemented sodium sulphide solution. The digestion period was 20 hr. at 20°C. in each experiment.

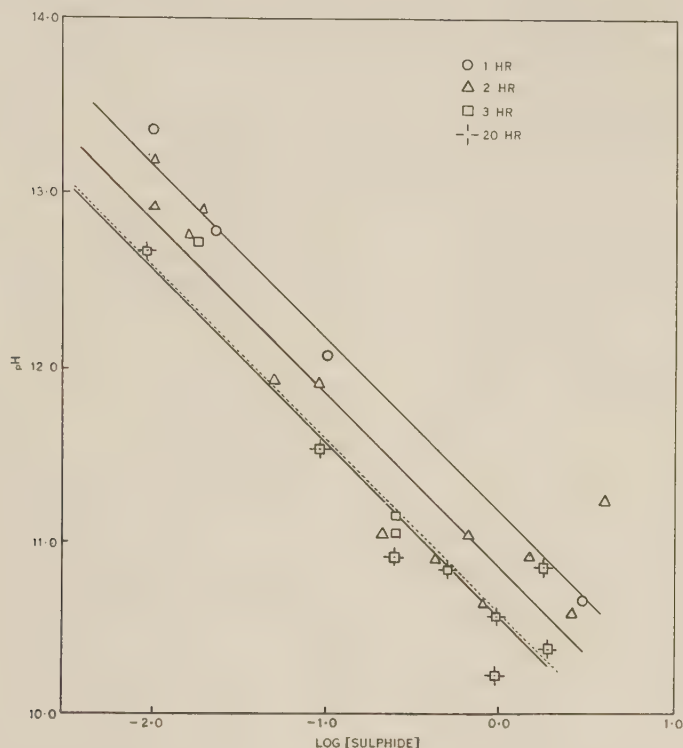


Fig. 10.—Relationship between the limiting concentrations of hydrosulphide ions and hydroxyl ions capable of reducing the strength of wool to zero.

It is evident that conditions of sulphide concentration and pH normally causing almost complete disintegration of the wool (\times) produced only partial disintegration or merely softening of the fibres (Δ or \square). However, after holding at room temperature for several days, in most instances the wool gradually softened and approached the expected equilibrium conditions. It is probable therefore that differences in the behaviour of potassium and sodium sulphide are attributable largely to slower diffusion of the more hydrated sodium ions into the wool fibre. This would have the triple effect of reducing the rate of diffusion into the wool of hydrosulphide and hydroxyl ions, with a concomitant lessening of hydration of the fibres by an osmotic effect. The latter effect would be increased by adding sodium chloride. The rate of depilation is decreased in a similar manner by high concentrations of the depilatory (Fig. 5).

Reduction of wool by sulphides involves oxidation of the sulphide to persulphide or some secondary oxidation product. The effect of persulphide on reduction of wool was determined by adding sufficient sulphur to the sulphide solutions to produce approximately equal concentrations of sulphide and persulphide. These solutions were included in an experiment of the usual type and in Figure 11 the results are compared with those given by sodium sulphide solutions. It is apparent that persulphide does not lessen the weakening of wool by sulphide but rather enhances it slightly, except at sulphide concentrations greater than 0.1M.

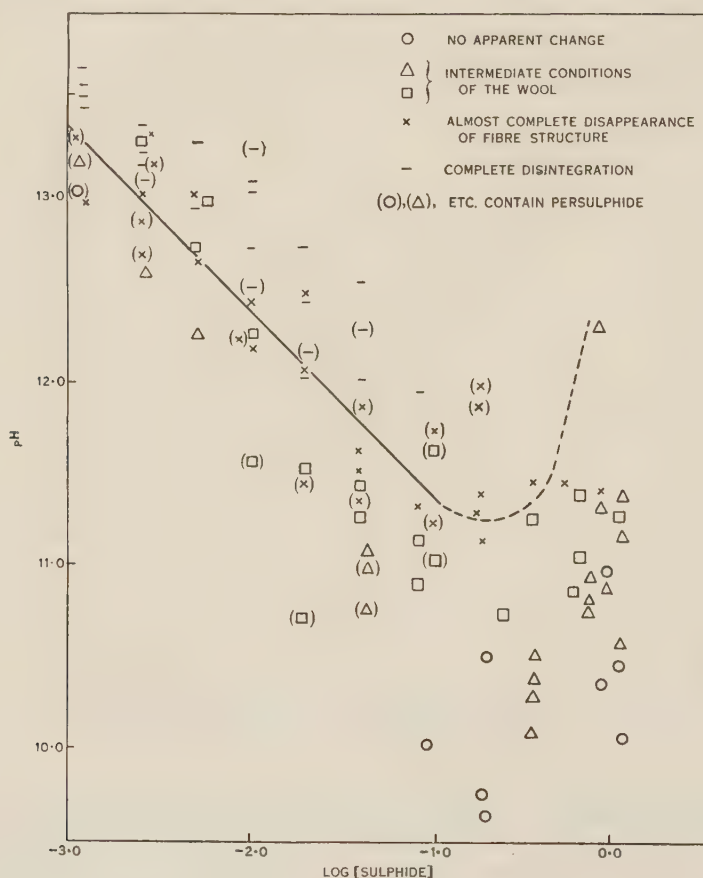


Fig. 11.—Effect of persulphide on the disintegration of wool by sodium sulphide. The curve represents the points denoted by \times .

From Figure 9 it follows that, over a limited pH range, the concentration of sulphide and the pH of solutions causing a constant degree of disintegration of wool fibres may be related by the expression

$$\text{pH} = -\log [\text{sulphide}] + C, \quad \dots \dots \dots (1)$$

which, if activity coefficients are neglected, may be rewritten

$$[\text{OH}^-] [\text{sulphide}] = C_1. \quad \dots \dots \dots (2)$$

Possible explanations for this relationship fall into three main classes: (1) Higher alkalinity may increase the accessibility of disulphide bonds to sulphide; (2) Hydroxyl ions, by rupturing salt links and hydrogen bonds, may contribute directly to the weakening of wool fibres (Blackburn and Lindley 1948); (3) Hydrogen or hydroxyl ions may have a direct role in the reduction of disulphide linkages by sulphides. It is probable that more than one of the hypotheses to be examined are operative simultaneously, but since a simple relationship exists between pH and $\log [\text{sulphide}]$ over a restricted range of these values, it is almost certain that over this pH range one effect preponderates to such an extent that secondary reactions may be neglected.

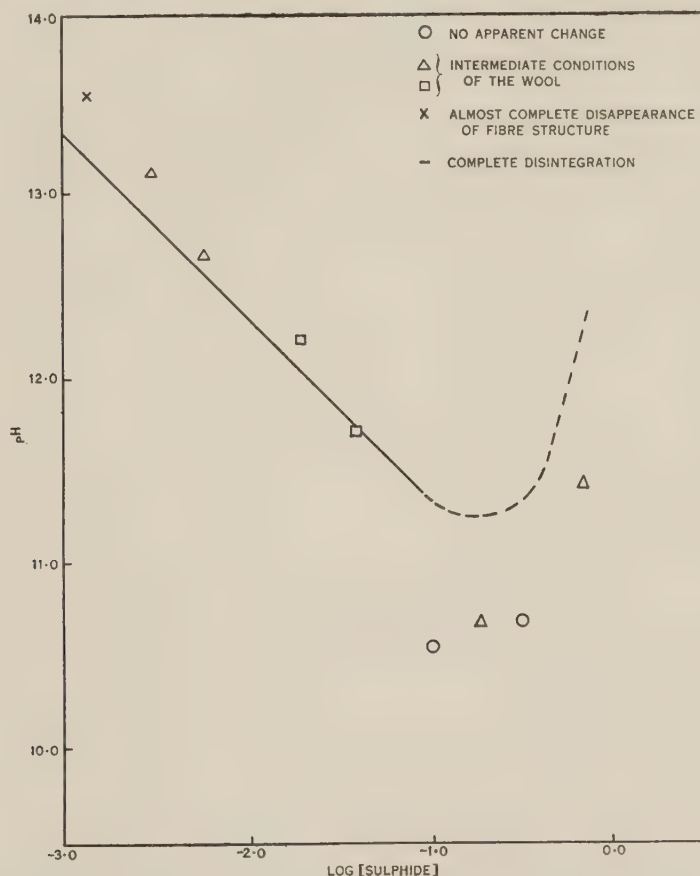


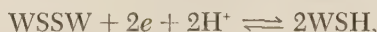
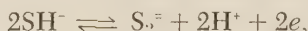
Fig. 12.—Effect of 2M sodium chloride on the disintegration of wool by sodium sulphide. The curve represents that normally joining points denoted by ×.

If, as the first hypothesis suggests, the rate of diffusion of sulphide into the wool fibres were increased by increasing pH, the slope of the curve relating pH and sulphide concentration in solutions bringing about a constant degree of disintegration of wool would vary with time, and as the system approached equilibrium the slope would approach infinity. This is not substantiated by

experiment (Fig. 9). It might also be suggested that dithiol linkages are inaccessible to hydrosulphide ions until certain hydrogen bonds or salt linkages are split. However, this could not be a satisfactory explanation if a dynamic equilibrium exists between hydrogen bonds or salt linkages and the groups contributing to their formation. The ease with which such linkages are broken and reformed indicates that such an equilibrium exists (Blackburn and Lindley 1948).

The second hypothesis suggests that sulphide and hydroxyl ions act independently on the wool fibre, the former rupturing dithiol bonds, the latter breaking hydrogen bonds or salt linkages. It is, in fact, possible but highly improbable that the equations relating the decrease in strength of wool to sulphide concentration and hydroxyl ion concentration, respectively, should be such that the effects of these ions are reciprocal.

The third hypothesis suggests that pH influences the reaction between disulphide bonds of wool and sulphide. If the oxidation of sulphide and reduction of wool dithiol bonds take place according to the equations



the following electrode equations hold at equilibrium:

$$E_h = E_1^\circ - \frac{RT}{2F} \ln \frac{[\text{SH}^-]^2}{[\text{S}_2^{2-}]} + \frac{RT}{F} \ln [\text{H}^+], \quad \dots \dots (3)$$

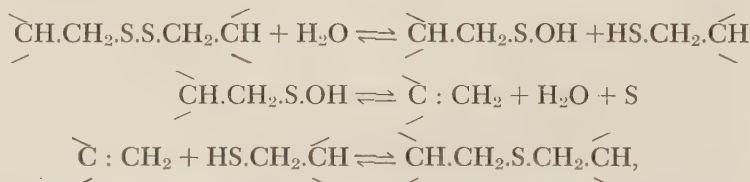
$$E_h = E_2^\circ - \frac{RT}{2F} \ln \frac{[\text{WSH}]^2}{[\text{WSSW}]} + \frac{RT}{F} \ln [\text{H}^+], \quad \dots \dots (4)$$

where [WSH] and [WSSW] represent the concentrations of wool thiol and dithiol groups respectively.

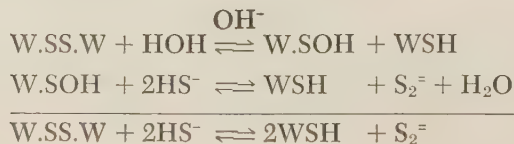
When both systems are present in equilibrium, therefore,

$$\frac{[\text{SH}^-]^2}{[\text{S}_2^{2-}]} = \frac{[\text{WSH}]^2}{[\text{WSSW}]} \text{antiln} \frac{2F}{RT} (E_1^\circ - E_2^\circ). \quad \dots \dots (5)$$

Cuthbertson and Phillips (1948) have shown that dithiol linkages in alkaline solution react as follows:



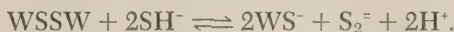
the stable lanthionine linkage being formed in this way. Bowes (1949) suggests that in strongly reducing solutions the group $=\text{CH}.\text{CH}_2.\text{SOH}$ is reduced to $=\text{CH}.\text{CH}_2.\text{SH}$, thus preventing the formation of the stable lanthionine linkage. The overall reaction with sulphide as reducing agent would then be represented by



Since the final equation does not differ from that used in deriving (5), the equilibrium state would again be described by this equation. The results cannot therefore be explained by a catalytic role of OH^- .

Vago (1937) suggested that the effect of pH can be explained in terms of the decrease in Eh of sulphide solutions with increase in pH. However, the possible effect of pH on the thiol system of keratin was not considered and, unless it is postulated that one of the components of this system is involved in a secondary reaction with H or OH^- , the Eh of the wool thiol system would also decrease with increasing pH, and the equilibrium condition would again be described by (5).

The pK values of the thiol groups of reduced wool are not known. However, the pK values of the thiol groups of cysteine, glutathione, and thioglycollic acid are known to approximate to 9.0 and it seems probable therefore that the thiol groups of reduced wool should have pK values of that order. These thiol groups would then be almost completely ionized over the pH range 11.0 to 13.5 and the reactions taking place would be described by the equation



If the total concentration of sulphhydryl groups in the wool, $[\text{WSH}] + [\text{WS}^-]$, is represented by $[\text{WR}]$,

$$K_{\text{WSH}} = \frac{[\text{WS}^-][\text{H}^+]}{[\text{WSH}]}$$

$$\text{and } [\text{WR}] = [\text{WSH}] + [\text{WS}^-]$$

$$\text{Therefore } [\text{WSH}] = \frac{[\text{WR}][\text{H}^+]}{K_{\text{WSH}} + [\text{H}^+]}. \quad \dots \quad (6)$$

Eliminating WSH from (5) and (6) and converting to logarithms, we obtain

$$-\log [\text{H}^+] = -\log [\text{SH}^-] - \log ([\text{H}^+] + K_{\text{WSH}}) + \frac{1}{2} \log \frac{[\text{WR}]^2 \text{S}_2^{2-}}{\text{WSSW}} + \frac{0.4343F}{RT} (E_1^\circ - E_2^\circ). \quad \dots \quad (7)$$

For values of H^+ considerably smaller than K_{WSH} , the term

$$-\log [\text{H}^+] - \text{pH}_s^* = -\log [\text{SH}^-] + \frac{1}{2} \log \frac{[\text{S}_2^{2-}][\text{WR}]^2}{[\text{WSSW}]} + \frac{0.4343F}{RT} (E_1^\circ - E_2^\circ) + \text{p}K_{\text{WSH}}. \quad \dots \quad (8)$$

Since the amount of sulphide oxidized would be equivalent to the amount of wool reduced, restriction of (8) to a constant degree of wool weakening would lead to constant values of $[\text{S}_2^{2-}]$, $[\text{WSSW}]$, $[\text{WR}]$, E_1° , E_2° , and $\text{p}K_{\text{WSH}}$, so that the expression, apart from variation in activity coefficients, represents a linear relationship between pH_s and $\log [\text{HS}^-]$ with a slope of -1 .

During the disintegration of wool there will also be a free energy change due to the increased homogeneity of the system and the equilibrium state would be influenced accordingly. However, if the rupture of dithiol linkages is the major cause of wool disintegration at high pH values, it is reasonable

* Using the notation of Ogston (1947).

to assume that the decrease of free energy due to increased homogeneity of the system can be expressed as a function of $[WR]$. It would then be possible to correct for this effect by including a term $\phi ([WR])$ in (4) and (8). Since $[WR]$ is constant for a constant degree of wool disintegration $\phi ([WR])$ would also be constant.

A more rigorous treatment with the inclusion of activity coefficients in (5) and (6) gives an expression for $-\log (H^+)$ similar to (8) but with the additional term

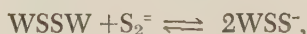
$$\frac{1}{2} \log \frac{f_{WS^-}^2 \cdot f_{H^+}^2 \cdot f_{S_2^-}}{f_{SH^-}^2 \cdot f_{WSSW}}$$

on the right hand side.

It is difficult to assess the possible influence of variation of activity coefficients on the linearity and slope of the curve. However, in considering solutions described by (8) it should be appreciated that, with decreasing ionic strength due to potassium sulphide, the ionic strength due to potassium hydroxide increases, a considerable concentration of potassium ions being built up during pH adjustment because of the buffer capacity of wool and the sulphide solution. Furthermore, the pH values obtained using the glass electrode would also be influenced by the activity coefficients of other ions in solution.

The curve obtained by assuming pK_{WSH} to be 9.0, and arbitrarily substituting 1.55 for the constant terms of (8), is represented by the broken line in Figure 9. It is seen to approximate closely to the points representing almost complete breakdown of the wool, which are denoted by \times , except at values of [sulphide] greater than 1.0M, where high salt concentrations complicate the issue.

In deriving (8), it has been assumed that the persulphide formed by oxidation of hydrosulphide ions remains completely ionized at the pH of the experiment. Although hydrogen persulphide is a much stronger acid than hydrogen sulphide, this assumption is probably unjustified in the light of what little is known of the pK values of hydrogen persulphide (Küster and Heberlein 1905). On the other hand, the results presented in Figure 11 indicate that persulphide does not decrease the disintegration of wool by sulphide and, in fact, at high concentration, it slightly increases the disintegration. It must be concluded therefore that if persulphide is a major product of the reaction, it enters into a secondary reaction, which offsets the tendency for the reverse reaction to take place. This may be due to reaction between persulphide and wool dithiol linkages.



or to further oxidation of the persulphide by wool to other polysulphides. Whatever the reason for this effect, it is apparent that small changes in $[S_2^-]$ due to partial association to HS_2^- are unlikely to affect the degree of wool disintegration significantly.

By combining (3) and (8) or by direct substitution for [WSH] in (4), an expression is obtained for the relationship between Eh of solutions and the number of residual disulphide bonds thus:

$$Eh = E^{\circ}_2 - \frac{RT}{2F} \ln \frac{[WR]^2}{[WSSW]} + \frac{RT}{F} \ln K_{WSH} \dots \dots (9)$$

This equation, like (8), is restricted in its application to a system in which $[H^+]$ is considerably less than K_{WSH} . Again it is clear that, apart from variation of activity coefficients, for a constant degree of wool weakening the Eh of the solution would be constant since WR and WSSW would be constant. Vago (1937) states that the tensile strength of horse hair keratin was reduced by sulphide solutions having reduction potentials in the range -0.3 to -0.4 V., the Eh of solutions just producing complete disintegration of the hair being constant and independent of pH. A similar range of potentials is required for the depilation of sheep skins (Fig. 7). Any hypothesis providing a satisfactory explanation of the relationship between $[OH^-]$ and [sulphide] for a constant degree of wool weakening will necessarily also provide an explanation for the constancy of Eh of such solutions as reported by Vago. This observation therefore provides experimental verification for the relationship expressed in (2). It should be pointed out, however, that, in similar experiments conducted by Vago, the slope of the straight line relating pH and sulphide was -0.7 instead of -1.0 . Since only four points are plotted in Vago's figure and in view of the difficulties of such experiments it seems probable that -1.0 is nearer the correct value, though some deviation from unit slope or a straight line relationship may occur as a result of variation of activity coefficients. The direct estimation of sulphydryl groups formed in reduced wool would probably provide more precise data.

In conclusion, it may be stated that the linear relationship between pH and the logarithm of the sulphide concentration, for solutions in equilibrium with wool that has undergone a constant degree of reductive degradation, is adequately explained by assuming the sulphydryl groups of wool to have a pK value of approximately 9.0. The fact that the Eh of such solutions is constant and independent of pH is also adequately explained on this basis.

CORRIGENDA

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Opposite page 354, Plate 1

Owing to turning of the block, Figure 1 is at bottom right; Figure 2 at bottom left; Figure 3 at top right; and Figure 4 at top left position of the plate.

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Page 491. In the caption below Figure 2: *for* 0.75M. *read* 0.075M.

WHITE ROOT ROT OF RASPBERRIES

By G. C. WADE*

[*Manuscript received December 16, 1950*]

Summary

The disease known as white root rot affects raspberries, and to a less extent loganberries, in Victoria. The causal organism is a white, sterile fungus that has not been identified. The disease is favoured by dry soil conditions and high soil temperatures. It spreads externally to the host by means of undifferentiated rhizomorphs, and requires a food base for the establishment of infection. The spread of rhizomorphs through the soil is hindered by high soil moisture content and consequent poor aeration of the soil.

The known natural host range of the fungus includes the raspberry, loganberry, and plum, but some other rosaceous plants have been infected artificially. It has not been recorded on any native plants. It is spread from one plantation to another by planting infected canes, and is spread within a plantation by root contact and movement of infected root and cane material during cultivation.

I. INTRODUCTION

The disease referred to by Victorian raspberry growers as white root rot has been known in that State for almost 50 years, and was first described in 1897 (McAlpin). No other references to the disease have been published though it was the subject of an unpublished paper by Miss Halsey, who studied the disease at the University of Melbourne.

Commercial raspberry growing in Victoria is now practically confined to a relatively small area east of the Dandenong Ranges, and the disease is present throughout the district. In some plantations it has spread rapidly, and a few have been practically wiped out in three or four years, but usually it spreads slowly and only a few bushes are killed each year.

The disease has recently (January 1950) been recorded from one plantation in the Molesworth district of Tasmania, but a survey of raspberry plantations in that State did not reveal any other cases. No similar trouble has been reported outside Australia.

The investigations described here were conducted during the years 1941 to 1947.

II. SYMPTOMS OF THE DISEASE

The disease is characterized by rotting of the roots and cane bases below ground level. The affected regions are covered with a dense mycelial mat, which is predominantly white in colour, though portions become cream-coloured

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and very compact and smooth in texture. Conspicuous, white rhizomorphs develop on the surface of the affected areas. The fungus does not attack the plant above ground level.

The first symptom on the above-ground portions of the plant is usually a yellowing of the foliage, accompanied by premature autumn tints. Shortly after the development of these symptoms, the affected portion of the bush wilts rapidly and dies. Sometimes, however, wilting and death may occur without any preliminary symptoms. When young plants are attacked, the whole bush dies simultaneously, but old bushes die gradually, a few canes at a time.

III. THE CAUSAL ORGANISM

(a) *Isolation and Proof of Pathogenicity*

A white fungus, which remained sterile in culture, was isolated consistently by planting out pieces of infected roots on potato dextrose agar (P.D.A.), the pieces being surface-sterilized with 1 in 1,000 mercuric chloride and then washed with sterile water.

Many infection experiments, which will be described in detail later in this paper, have been conducted and typical symptoms of the disease produced by inoculation with a soil culture of the organism. The same fungus has been re-isolated from the inoculated plants, thus proving that it is the cause of the disease.

(b) *Morphology of the Fungus*

In culture on P.D.A. the fungus produces a white mycelium, which grows fairly rapidly, producing a colony 8 cm. in diameter in 10 days at 25°C. The advancing edge of a young colony is even with a silken sheen, while the central portions are white and appressed. As the colony ages the edges develop a brownish tint, and portions of the central area become compressed into a flat, felty layer with a cream coloration.

The surface of this compressed layer bears highly branched structures consisting of hyphae about 0.5 to 1 μ in diameter. The structures are variable in size and range from $10 \times 15 \mu$ to $30 \times 50 \mu$. They are one of the most characteristic features of the fungus and are illustrated in Figure 1. They do not stain readily, but several hours in Harris's haematoxylin is effective.

The hyphae of the fungus are of two types. Young colonies consist entirely of septated hyphae, 2.5 to 5.2 μ (average 3.8 μ) in diameter. Older colonies consist of the same type of hyphae, mixed with very fine hyphae, 0.4 to 1 μ in diameter. These fine hyphae predominate in the compressed portions that develop as colonies age.

The fungus produces white, undifferentiated rhizomorphs on its natural host, in soil culture, and occasionally on agar media. They are 0.5 to 1 mm. in diameter and similar in type to those produced by *Fomes lignosus* and classified by Garrett (1944) as type 5.

No fruiting stage of the fungus has been found either in the field or on artificial media. For that reason the fungus has not been identified. Material was forwarded to the Commonwealth Mycological Institute, but they were unable to identify it.

McAlpin (1897) stated that *Hypholoma fasciculare* (Huds.) was the cause of the disease, but cultures made from sporophores of that fungus bore no close resemblance to the causal organism of white root rot.

Though the general appearance of cultures of the white root rot fungus resembles those of a Basidiomycete, no clamp connections have been found, so that it has not been proved to belong to that group.



Fig. 1.—Freehand drawing of highly branched structures of the white root rot fungus.

(c) *Physiology of the Fungus*

(i) *Growth on Culture Media*.—The fungus grows readily on potato dextrose agar, malt agar, or raspberry cane extract agar. The media rapidly become brittle and numerous octahedral crystals develop. It grows very sparsely on synthetic media such as Czapek's solution, unless thiamin is added. In a typical experiment Czapek's solution (formula as given by Riker and Riker 1936) was prepared and thiamin added to half the solution at a concentration of 20 μ g./l. Fifty ml. of the solutions were distributed into 250 ml. erlenmeyer flasks and six of each solution were inoculated. The fungus grew rapidly on the flasks with thiamin, but scarcely any growth occurred on those without thiamin, even with prolonged incubation.

(ii) *Temperature*.—To determine the optimum temperature for the growth of the fungus P.D.A. plates were inoculated at the centre with a piece of culture about 1 mm. in diameter. Six plates were incubated at each of the temperatures shown in Table 1 for five days, when the diameters of the colonies were measured. These results indicate that the optimum temperature for the growth of the fungus is about 27°C.

IV. EFFECT OF THE FUNGUS ON THE PLANTS

Infection by the fungus is confined to the underground portions of the plant. However, extensive growth of the fungus may be present on the main root without causing death, and death does not usually occur until a large proportion of the fibrous roots have been destroyed.

Sections of fibrous roots, main roots, and of canes at ground level, 1, 3, and 6 in. above ground level were cut and stained by Haidenhain's haemotoxylin. The sections showed that attack commences on the outside of the root, and then grows into the inner tissues, causing general rotting of all tissues. There was no evidence that the fungus spread internally in the vascular tissue, and it was not detected in the above-ground portions of the plant.

TABLE 1
EFFECT OF TEMPERATURE ON GROWTH OF THE WHITE ROOT ROT FUNGUS

Temperature	19°C.	21°C.	23°C.	25°C.	27°C.	29°C.
Mean colony diameter	3.7 cm.	4.3 cm.	5.2 cm.	5.6 cm.	6.6 cm.	6.1 cm.

V. FACTORS INFLUENCING INFECTION

(a) *Method of Inoculation*

Early infection experiments with pure cultures of the fungus grown on sterile soil to which dextrose and peptone were added were only occasionally successful, while inoculations with naturally infected root material were successful. It was then found that pure cultures produced infection if a piece of raspberry cane was added to the culture, and therefore experiments were undertaken to determine whether the fungus requires a "food base" to establish infection.

(i) *Pot Experiments.*—Lloyd George raspberry canes were planted in virgin red mountain soil in glazed porcelain crocks. Of these, 24 were inoculated with cultures on 500 g. of sieved soil plus 1 g. dextrose and 0.1 g. peptone, 24 with similar cultures to which a piece of sterile raspberry cane had been added when the medium was prepared, 24 with soil cultures to which sterile raspberry canes had been added one week before inoculating the plants, and a further 24 were left uninoculated as controls. A mulch of grass clippings was added to half of each group to determine the possible effect of organic matter on infection.

To avoid the effect of position, each series of 12 pots was divided into three groups of four pots, and the groups randomized. The first experiment was conducted in 1945-46 and inoculations made on January 5. It was repeated the following season and inoculated on November 27.

The plants were observed throughout their growing period and a record was kept of all deaths from white root rot. On May 29, 1946, and on April 10, 1947, all surviving plants were pulled up, and examined for evidence of infection.

In both seasons no uninoculated plants, or plants inoculated with a soil culture without the addition of raspberry cane material, became infected with white root rot, but infections occurred when the inoculations contained a piece of raspberry cane. The progressive development of infection is shown in Table 2.

TABLE 2
EFFECT OF METHOD OF INOCULATION ON INFECTION WITH WHITE ROOT ROT

Method	Added Material	Deaths from White Root Rot			Infections Noted at Conclusion	Total Infection in 12 Plants
		Jan.	Feb.	Mar.		
1945-46						
Soil culture (Method A)	No addition	0	0	0	0	0
	Grass clippings	0	0	0	0	0
Raspberry cane added when soil culture prepared (Method B)	No addition	0	1	1	1	3
	Grass clippings	0	0	1	4	5
Raspberry cane added to soil culture one week prior to inoculation (Method C)	No addition	0	2	1	2	5
	Grass clippings	0	0	1	4	5
1946-47						
Method A	No addition	0	0	0	0	0
	Grass clippings	0	0	0	0	0
Method B	No addition	3	1	0	2	6
	Grass clippings	1	0	0	1	2
Method C	No addition	1	1	0	5	7
	Grass clippings	0	1	0	3	4

The most important result was to demonstrate that, under the conditions of these experiments, infection could not be established without the presence of a "food base." There are several examples in the literature of other root disease fungi behaving in the same way, and this literature has been discussed by Garrett (1944). Bliss (1941) found that *Armillaria mellea* was unable to establish infection unless the rhizomorphs were in contact with a food base, and results of Petch (1921, 1928) and of Tunstall (1930), as quoted by Garrett (1944), showed that *Fomes lignosus* and *F. noxious* behave similarly.

The stage at which the food base material was added to the culture did not appear to affect the infectivity of the inoculum. Although the addition of

organic matter did not prevent infection, it appeared to delay the death of infected plants. It was noted that the addition of grass clippings markedly increased the root development of the plants. Infected plants do not die until a considerable proportion of the fibrous roots have been destroyed, so that any factor that increased root development could be expected to delay death.

As no infection developed on plants inoculated with a soil culture of the organism, and receiving a dressing of grass clippings, it is apparent that grass clippings will not replace raspberry cane material as "food base." A further experiment was conducted to determine whether the "food base" could be replaced with a simple carbohydrate, with or without addition of vitamins. A single Lloyd George raspberry was planted in red mountain soil in each of 36 porcelain crocks. Sucrose at the rate of 5 cwt. per acre was added to 12 crocks, and sucrose at 10 cwt. per acre plus ground yeast at $\frac{1}{2}$ cwt. per acre to a further 12 pots. These crocks were inoculated with a soil culture of the organism, and the 12 untreated pots with a soil culture containing raspberry cane material. No plants became infected in the first two treatments, while six of the plants inoculated with raspberry cane material developed white root rot. Therefore, sucrose, with or without vitamins, cannot replace the natural "food base."

(ii) *Field Experiments.*—To determine whether similar results would be obtained under field conditions, an experimental plot of Lloyd George raspberries was planted in virgin soil in August 1945. The method of inoculation already described and the effect of organic matter were tested when the plants were established. There were three replicates, each consisting of two rows of 12 plants of each method of inoculation and of uninoculated raspberries. A guard row of uninoculated raspberries was left between the paired rows of inoculated plants. Tick beans were sown in one row of each pair in May 1946, and were hoed in as a source of organic matter in October.

The inoculations were made on December 4, using cultures prepared in the manner described previously. A hole was dug with a trowel within four inches of a root near the crown of the plant and the culture added and covered with an inch of soil.

The experiment was kept under observation until the author left Victoria in April 1947. Up to that time no plants died, but examination of the roots near the sites of inoculation showed that infection had become established on a number of plants that had received inoculations containing raspberry cane material.

On May 20, 1948, the experiment was examined by Mr. C. R. Millikan, Senior Plant Pathologist of the Victorian Department of Agriculture, who recorded the number of plants that had died of white root rot and kindly supplied the figures shown in Table 3.

The results were examined statistically by Mr. G. A. McIntyre, Division of Mathematical Statistics, C.S.I.R.O., who reported that the addition of organic matter had no significant effect on the results, treatment 3 differed from treatment 4 ($P < 0.001$) and treatments 1 and 2 differed from treatments 3 and 4.

The main conclusions of the pot experiments were therefore confirmed in the field. In the field, however, the addition of a food base was more effective if the fungus was well established on it before inoculation of the plants.

TABLE 3
DEATHS FROM WHITE ROOT ROT IN GROUPS OF 12

Method of Inoculation	No Addition			Organic Matter		
1. Uninoculated	0	0	0	0	0	0
2. Soil culture	0	0	0	0	0	0
3. Raspberry cane added when soil culture prepared	12	12	11	11	12	12
4. Raspberry cane added to soil culture one week prior to inoculation	9	8	9	12	8	9

(b) Influence of Soil Moisture Content

(i) *On Infection of Growing Plants.*—Field observations suggested that the disease was more severe in dry, exposed situations. An experiment was therefore conducted to determine the effect of soil moisture on infection with the white root rot fungus.

Waxed canisters 12 in. in diameter and 15 in. deep were filled with a weighed amount of red mountain soil of known moisture content. Lloyd George raspberry canes were planted in August 1945 and one month later were inoculated by the addition of a culture of the white root rot fungus on sterile soil mixed with raspberry cane material. They were then divided into three groups of 16. They were watered twice weekly to constant weight, so that if uniformly wet the soil of one set would have been at 50 per cent. of the water-holding capacity, another at 70 per cent., and the third at 90 per cent. of the water-holding capacity. The water-holding capacity of the soil was determined by the method described by Riker and Riker (1936). Hendrickson and Veihmeyer (1941) have pointed out that soil does not become uniformly moist when water is applied to the surface. Therefore the three series are better described as dry, medium wet, and wet soil series.

The pots were sheltered by a canopy of a transparent fabric to keep rain from wetting the soil. The 16 canisters in each series were divided into four groups and randomized to overcome chance effects due to position. The dates when plants died of white root rot were recorded and in May 1946 the remaining plants were pulled up and examined.

All the plants in the dry soil series died from the disease, the first death occurring on December 4 and the last on February 13. One plant in the

medium wet series died on February 5, but no plants in the wet series died of white root rot, though three plants died through waterlogging when they were accidentally overwatered by exposure to rain.

When the surviving plants were examined it was found that three plants in the medium wet series and one plant in the wet series showed infection of the tap root only, but the fibrous roots were uninfected. These results demonstrate that infection is inhibited if the soil is kept close to saturation with water, but develops readily if the soil is kept relatively dry. They also suggest that the disease only kills the plants when general root infection, involving the fibrous roots, develops.

(ii) *Effect of Soil Moisture on Growth of the Fungus in Soil Culture.*—The results of the experiment just described suggested that the soil moisture content may influence the growth of the fungus through the soil. Therefore a laboratory experiment was conducted to determine if that was correct.

Cylindrical glass jars 8.5 cm. wide and 11 cm. deep were filled with a weighed amount of red mountain soil of known moisture content. Water was then added to bring the moisture content of five jars to 30, 50, 70, and 90 per cent. of water-holding capacity. The soil was then stirred thoroughly. The jars were inoculated by inserting a piece of raspberry cane, on which the fungus had been well established, in a vertical position in the centre of each jar. Two clean glass slides were then pushed vertically into the soil on opposite sides of each jar.

TABLE 4
EFFECT OF SOIL MOISTURE CONTENT ON GROWTH OF THE WHITE ROOT ROT FUNGUS IN SOIL

Percentage of Water-holding Capacity	Time Taken for Rhizomorphs to Travel 4 cm. (days)	Depth of Penetration into Soil (cm.)
30	24	4
50	36	4
70	49	1
90	*	0.5

* In one of the five replicates rhizomorphs travelled 4 cm. in 45 days, but in the others no rhizomorphs travelled that distance during the period of the experiment.

The jars were incubated at 25°C. and the weight restored by spraying with water twice weekly. They were observed daily and the time taken for rhizomorphs to reach the sides of the jars was noted. After eight weeks the slides were removed, loosely adhering soil particles rinsed off, and stained with dilute carbol-erythrosin. They were examined microscopically, and the depth of penetration of the fungus measured. The slide burial method employed was based on that of Rossi-Cholodny as described by Blair (1945).

The results are shown in Table 4, the figures being the mean of five replicates. The experiment showed that rhizomorphs of the white root rot fungus travel more rapidly through dry soil and penetrate more deeply than in moist soil. This at least partly explains the greater development of the disease in dry soil. It seems probable that high moisture content reduces soil aeration, and the fungus is inhibited through carbon dioxide accumulation and lack of oxygen.

Some support for this suggestion was obtained when cultures of the fungus, placed in an incubator in which an orange storage experiment was in progress, failed to grow. Analysis of the air in the incubator showed that it contained 12 per cent. CO₂. Cultures made at the same time, and placed in another incubator, grew normally. Cultures of *Penicillium italicum* and *P. digitatum*, which were in the incubator containing 12 per cent. CO₂, grew normally.

Very few raspberry plantations in Victoria are irrigated, and there was no opportunity of testing the effect of irrigation at regular intervals on the incidence of the disease. The few irrigated plantations observed were practically free from the disease. It is not troublesome in sheltered, low-lying situations where the soil remains moist throughout the summer, and the most serious outbreaks of the disease were noted in exposed, dry situations. The only Tasmanian record occurred in soil that frequently became very dry during the summer months.

(c) Effect of Soil Temperature

As shown in Section III (c) (ii), the raspberry white root rot fungus has a high optimum temperature. To determine whether infection of raspberries would develop more rapidly at high rather than low temperatures, unsterilized red mountain soil was placed in metal canisters and inoculated with cultures of the white root rot fungus grown on sterile soil plus raspberry cane material.

Raspberry canes of the Lloyd George variety were placed in the canisters on August 14, 1946. The canisters were then placed in temperature tanks of the Wisconsin type and eight canisters were held at 28°C. and eight at 18°C.

On October 26, five plants at 28°C. showed signs of wilting, but all the plants at 18°C. appeared healthy. On November 30, seven of the eight plants at 28°C. and one plant at 18°C. died of white root rot. The experiment was continued for several weeks, but no further deaths occurred and examination of the roots did not reveal any further infections.

The disease is, therefore, favoured by high temperatures.

(d) Effect of Lime

A small-scale pot experiment was conducted in which red mountain soil of pH 5.6 was placed in porcelain crocks. Slaked lime was added to 12 pots at the rate of 1 ton per acre and to 12 pots at the rate of 4 tons per acre, while no lime was added to a further 12 pots. The pots were inoculated with the white root rot fungus, and Lloyd George raspberries planted.

Two plants in each series developed white root rot and died. Although the amount of infection was not high in this experiment, the results suggest that alteration of soil pH by addition of lime does not have a marked influence on the disease.

VI. METHOD OF SPREAD IN THE FIELD

The white root rot fungus has not been detected on native plants, and is apparently introduced into new plantations on virgin soil entirely by planting canes obtained from infected raspberries.

In 1945 an experimental plantation of 1½ acres of Lloyd George was laid down under the author's supervision on virgin soil in the Silvan district. All the canes were examined carefully before planting, and any that showed the presence of the white root rot fungus were discarded. No fungicidal treatment was applied to the remaining canes, as it was considered unlikely that surface contamination with the mycelium of the fungus would produce infection. The plot was examined frequently and only one plant of a total of 2,000 canes developed the disease.

The disease may also be introduced by replanting on soil that has recently carried infected raspberries, and still contains infected root material. If white root rot becomes established in a plantation, subsequent spread takes place more rapidly along the rows than across from one row to the next.

Detailed examination of the spread of infection from one plant to its neighbour has shown that it usually takes place by root contact. As raspberries have an extensive root system, roots of neighbouring plants intermesh, and contact between infected and healthy roots occurs. Rhizomorphs of the fungus then pass from the diseased to the healthy root. No evidence was obtained that rhizomorphs can spread more than four inches from the food base. Most raspberry growers practise deep ploughing between the rows, thus preventing lateral spread of roots near the soil surface. This reduces the infection across rows since the fungus requires good soil aeration and would be less likely to spread along deep roots spreading across from row to row, than along surface roots between plants in the one row.

New foci of infection become established by carrying pieces of infected root and cane base material along the rows during cultivation. If this material lodges near raspberry roots, rhizomorphs developing from it produce infection. Subsequent spread to adjacent plants then takes place in the manner already described.

VII. HOST RANGE

Under natural conditions this disease is a serious trouble only on raspberries, but it also affects loganberries. However, the disease does not kill infected loganberries rapidly, and no serious losses have been noted. One case of a root and crown rot due to a fungus morphologically identical to the white root rot fungus was observed on plums on cherry plum (?) stocks at Wandin, Vic. The plums had been planted immediately after a severely infected patch of raspberries had been removed. When observed the plums were 12 years old and three trees showed infection. The disease has not been seen on any other host in the field.

To determine whether any other rosaceous plants could become infected with the disease by inoculation, six plants of apples, pears, peaches, apricots,

blackberries, cherries, plums, *Rosa multiflora*, *Rosa noya*, and the native raspberry (*Rubus parvifolius*) were planted in earthenware pots. They were inoculated with a culture of the white root rot fungus grown on sterile soil plus raspberry cane material on November 18. No plants died and on the following April 10 the plants were pulled up and examined.

Infection was present on four plants of *Rosa multiflora*, one plant of *Rosa noya*, five apples, three pears, and three cherries. However, the infection had been arrested in all cases and plants of those species appeared to be naturally resistant to infection. Plums, peaches, apricots, and the native raspberry were entirely free from infection. All the blackberries were infected with the disease though the plants were not killed. However, natural infection of blackberries has not been observed in the field.

VIII. DISCUSSION

Apart from one record in Tasmania, white root rot of raspberries has not been reported outside Victoria, and it seems probable that few raspberry-growing districts would provide the conditions that favour the development of the disease. The necessary factors are low soil moisture content and high soil temperature. The Victorian summer is hot and dry, and as few raspberry growers are able to irrigate, suitable conditions are present. It appears significant that the only case reported in Tasmania was in an exceptionally dry situation, and it was absent from more favoured portions of the same property.

Pot experiments showed that the disease is inhibited in soil of high moisture content, and at least part of this effect is probably due to reduced soil aeration. A similar explanation of the effect of high soil moisture content on certain other soil-borne diseases has been offered by other workers, e.g. Hull and Wilson (1947) in their work on factors influencing infection with *Helicobasidium purpureum*. In laboratory experiments it was found that high soil moisture content reduced the depth of penetration and rate of lateral spread of rhizomorphs. In investigations on the behaviour of *Rhizoctonia solani* in soil Blair (1943) found that it grew most rapidly through soil of the lowest moisture content tested and that its growth was accelerated by aeration of the soil.

Infection with raspberry white root rot can only be established if the fungus is growing on a food base, and inoculations with cultures on soil were completely ineffective. This has an important bearing on the control of the disease as there is little chance of spreading the disease through a plantation, except by distribution of pieces of infected root material during cultivation. If the plantation is inspected and all infected plants removed as completely as possible before cultivation, the disease will not spread, except to neighbouring plants. Soil sterilization should not be necessary because the movement of soil that contains the fungus not attached to root or cane material will not spread the disease. The spread of the disease by root contact to neighbouring plants can be avoided by completely removing an apparently healthy plant on either side of the infected one.

IX. ACKNOWLEDGMENTS

The work was conducted while the writer was a member of the Biological Branch, Department of Agriculture, Victoria. Miss P. Lawrence, of that Branch, gave valuable assistance with the laboratory and pot experiment work.

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Mr. G. A. McIntyre, of the Division of Mathematical Statistics, C.S.I.R.O., kindly made the statistical analyses of the data.

X. REFERENCES

- BLAIR, I. D. (1943).—Behaviour of the fungus *Rhizoctonia solani* Kühn in the soil. *Ann. Appl. Biol.* **30**: 118-27.
- BLAIR, I. D. (1945).—Technique for soil fungus studies. *N.Z. J. Sci. Tech.* A **26**: 258-71.
- BLISS, D. E. (1941).—Artificial inoculation of plants with *Armillaria mellea*. *Phytopathology* **31**: 859.
- GARRETT, S. D. (1944).—"Root Disease Fungi." (Chronica Botanica Co.: Waltham, Mass.)
- HENDRICKSON, A. H., and VEIHMEYER, F. J. (1941).—Moisture distribution in soil in containers. *Plant Physiol.* **16**: 821-6.
- HULL, R., and WILSON, A. R. (1947).—Distribution of violet root rot (*Helicobasidium purpureum* Pat.) of sugar beet, and preliminary experiments on factors favouring the disease. *Ann. Appl. Biol.* **30**: 118-27.
- MCALPIN, D. (1897).—*Dep. Agric. Vict. Guide to Growers* **32**: 10.
- PETCH, T. (1921).—"The Diseases and Pests of the Rubber Trees." (Macmillan and Co.: London.)
- PETCH, T. (1928).—The parasitism of tea root disease fungi. *Tea Quart.* **1**: 10-5.
- RIKER, A. J., and RIKER, R. S. (1936).—"Introduction to Research on Plant Diseases." (John S. Swift & Co.: St. Louis.)
- TUNSTALL, A. C. (1930).—Vegetable parasites of the tea plants. Blights on the root. *Quart. J. Indian Tea Ass.* **1930**: 28-36.

WHITE ROOT ROT OF RASPBERRIES

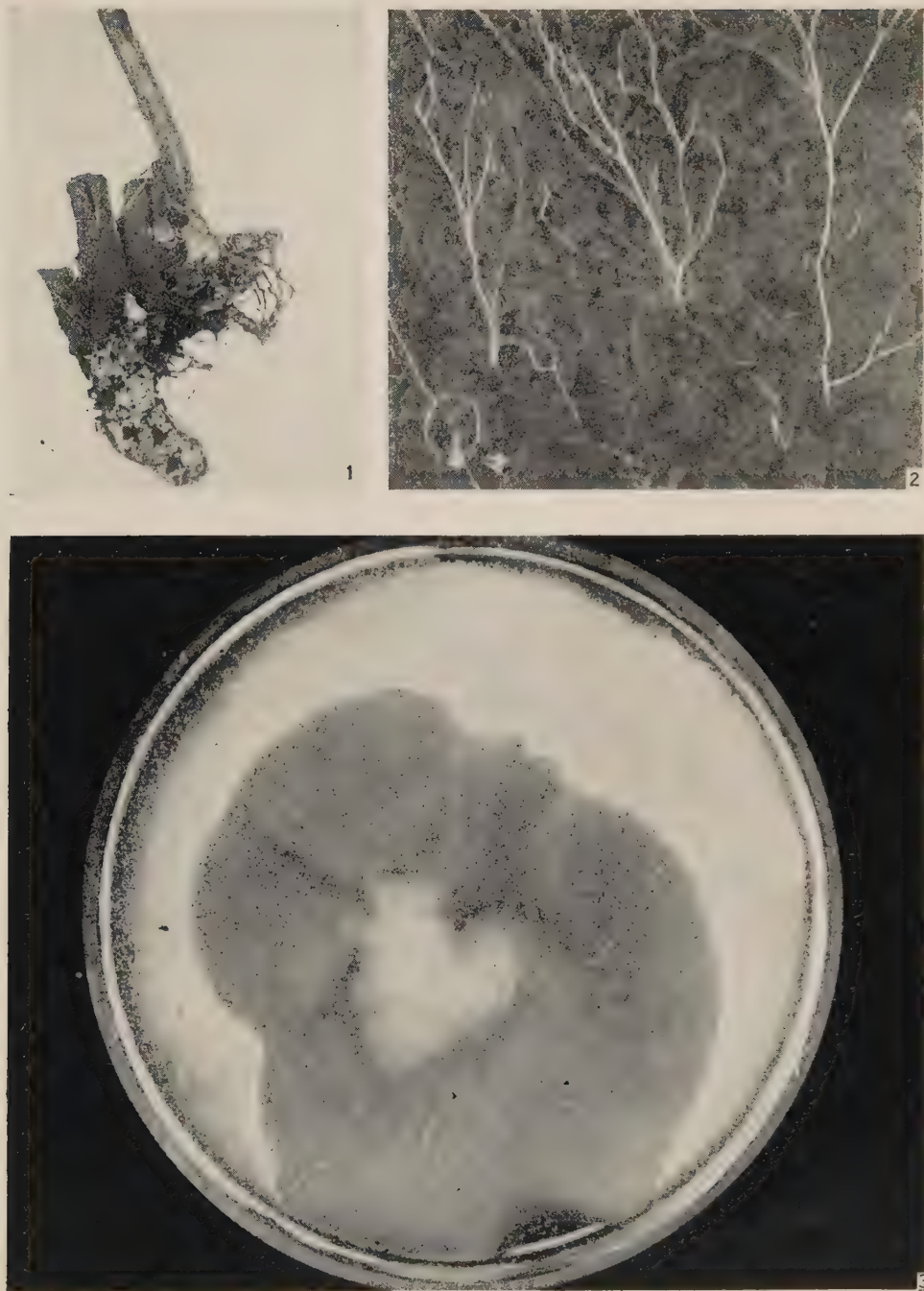


Fig. 1.—Raspberry root infected with white root rot.

Fig. 2.—Rhizomorphs of the white root rot fungus growing in soil culture.

Fig. 3.—Culture of the white root rot fungus on potato dextrose agar.

SOME STRAINS OF POTATO VIRUS X AND THEIR SPONTANEOUS MUTATION

By E. M. HUTTON* and J. W. PEAK*

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Summary

One virulent and three avirulent potato virus X strains were characterized qualitatively and quantitatively by the reactions of *Gomphrena globosa* and *Capsicum frutescens*. Spontaneous virulent mutants arose from time to time in the avirulent strains cultured in *Datura stramonium*. Avirulent strains developing low virus concentrations in their host plant had higher mutation rates than one developing a higher concentration in the host. Mutation rate appeared to be influenced by environmental conditions. The mutants from the three avirulent strains were similar and did not appear to differ from the ordinary virulent control strain. The mutants, owing to their greater biochemical vigour, almost entirely suppressed their parent avirulent strains.

I. INTRODUCTION

Previous genetical work on the reactions to virus X of potato seedlings derived from parents tolerant to this virus resulted in the separation of strains differing in virulence (Hutton 1948). Three of the avirulent strains, which were symptomless in *Datura stramonium* L. and produced only a fleeting mottle in *Nicotiana tabacum* L., were cultured in *D. stramonium* for over two years. At the same time one of the virulent strains was cultured in *D. stramonium* as a control. Opportunity was thus afforded to study some of the characteristics of these strains. During the two-year period spontaneous mutations occurred from time to time in each of the avirulent strains and observations were made on the rate and type of mutation.

Matthews (1949) has presented evidence to show that mottle-type strains of potato virus X usually have a relatively high forward mutation rate compared with severe ones, and he indicated also the possibility of back mutation from severe to mild strains. The present paper furnishes additional data to that of Matthews (1949) on mutation rates in strains of virus X that were apparently milder than those used by him. Further, the strains described in this paper were characterized both qualitatively and quantitatively by means of globe amaranth (*Gomphrena globosa* L.) as described by Wilkinson and Blodgett (1948) and also by *Capsicum frutescens* L. so that an indication of the inherent qualities of the strains leading to differences in rates of mutation was obtained. Globe amaranth proved also of use in the characterization of the mutants that arose from the avirulent strains.

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The vital constituents of both viral and nuclear material are chemically similar and both viruses and genes are subject to spontaneous mutations. In addition, X-rays induce mutations in viruses (Gowen 1939) as was found for flowering plants by Stadler (1928). It is thus evident that studies on the mutation of plant viruses could assist in an understanding of gene mutation in plants.

II. MATERIALS AND METHODS

The origin of the X strains used in this study has been mentioned already. The avirulent strains were designated 31-45, 50-57, and 50-58, and the virulent one 25-144. Over the two-year period of the experiments, each strain was transferred serially to a batch of six young *D. stramonium* plants at two- to three-weekly intervals. The inoculum for a transfer to a fresh batch of young plants was prepared by grinding composite leaf samples from symptomless plants of the previous batch with pestle and mortar at the constant proportion of 1 : 40 by weight with water. This was applied with a ground glass spatula after dusting the leaves with fine carborundum powder. After each transfer, the symptomless plants of the old batches carrying the avirulent strains were reinoculated with the virulent strain, and usually, protection was maintained in 50 per cent. or more of the plants of a batch thus proving the presence of the masked strains. The failure of complete protection by masked strains has been noted previously (Hutton 1948).

Wilkinson and Blodgett (1948) developed the use of globe amaranth for qualitative and quantitative work with potato virus X because of its opposite paired leaves, which are similar in appearance and sensitivity, and give well-defined local lesions without systemic infection. Preliminary tests with this plant showed it to be suitable for the study of some of the inherent characters of the virus X strains used in these experiments. To obtain optimum results with globe amaranth, the plants should have grown quickly and be used just prior to or at the commencement of flowering. Virus transfers to this species were made in the same way as those to *D. stramonium*.

Salaman (1938) showed that *Capsicum annuum* L. reacted to all the strains of potato virus X he isolated, including the masked one. It was thus of interest to compare the reactions of *Capsicum frutescens* L. to the four X strains with those of globe amaranth. Two varieties of *C. frutescens* were selected, World Beater No. 13 and an unnamed variety obtained from Professor C. M. Haenseler, Rutgers University, New Jersey, U.S.A.

III. CHARACTERISTICS OF THE FOUR X STRAINS AS INDICATED BY GLOBE AMARANTH (*GOMPHRENA GLOBOSA* L.)

Six pairs of opposite leaves representing young, medium, and old stages of development were selected on each of four well-grown globe amaranth plants. A randomized experiment was then done so that each X strain was inoculated to 12 leaves which represented a leaf from the three growth stages

on all four plants. To ensure accuracy, lesions were counted by artificial light eight days after inoculation. The results are tabulated in Table 1 and the differences between the X strains are depicted in Plate 1. It will be noticed in Plate 1 that the virulent strain, 25-144, tended to give better-defined lesions than the avirulent ones.

TABLE 1
MEAN NUMBER OF LESIONS ON LEAVES OF GLOBE AMARANTH FOLLOWING
INOCULATION WITH FOUR STRAINS OF POTATO VIRUS X

Strain of X	Avirulent		Virulent	
	31-45	50-57	50-58	25-144
Mean number of lesions per leaf	31.75	50.00	33.63	94.25

Minimum difference between treatment means for significance at 5 per cent. = 13.84, at 1 per cent. = 18.83.

The statistical analysis of the data presented in Table 1 was made on the results from leaves that were classified at the time of inoculation as being of old and intermediate age. The results from young leaves were too variable to be of value, so were not included. It will be observed in Table 1 that the virulent strain 25-144 produced from almost twice to three times the number of lesions of the avirulent strains on globe amaranth, the results being highly significant. There is no significant difference in mean number of lesions per leaf between the avirulent strains 31-45 and 50-58, and at the 5 per cent. level both of these strains produced significantly fewer lesions per leaf than 50-57. It is apparent from these results that the virulent strain existed in high concentration in *D. stramonium* and that significant inherent differences existed among the avirulent ones which attained a lower concentration in the host. The characteristics of the X strains determine their behaviour in host plants, and as will be indicated later, the differences between the avirulent ones affect their rate of mutation as well as their protective action against the ingress of a virulent strain.

IV. REACTIONS OF TWO VARIETIES OF SWEET PEPPER (*CAPSICUM FRUTESCENS* L.) TO FOUR X STRAINS

The three avirulent strains and one virulent strain of virus X were inoculated separately to different plants of the two sweet pepper varieties. Each strain was inoculated to a large leaf midway on three plants of each variety, the plants being 10-12 in. high and growing vigorously. The reactions on the inoculated leaves were observed after eight days and the symptoms in the uninoculated portions of the plants after 22 days. The results are summarized in Table 2.

It is evident that there is a correlation between these and the results obtained previously with globe amaranth and that a difference in type of reaction to virus X exists between the two sweet pepper varieties. In the variety

ex Haenseler none of the strains, including the virulent, produced necrosis, and growth, although mottled, approached normal, while abscission of the inoculated leaves occurred slowly. With World Beater No. 13, small necrotic spots in ill-defined rings appeared rapidly on the inoculated leaves, and the numbers and extent of the necrotic spots gave a virulence rating of the strains comparable to that obtained with globe amaranth. Abscission of the inoculated leaves in World Beater No. 13 was rapid, and the necrotic systemic symptoms were accompanied by marked growth depression, particularly with strains 25-144, 31-45, and 50-58.

It is apparent that certain sweet pepper varieties like World Beater No. 13 are useful in the characterization of X strains. Further, the necrotic reaction to virus X in sweet pepper appears to be genetically controlled.

TABLE 2
COMPARISON BETWEEN THE REACTIONS OF TWO SWEET PEPPER VARIETIES
(*CAPSIUM FRUTESCENS* L.) TO ONE VIRULENT AND THREE AVIRULENT STRAINS
OF VIRUS X

X Strain	Variety† ex Haenseler		World Beater No. 13	
	Inoculated leaf reaction after 8 days	Symptoms in leaves below growing point after 22 days	Inoculated leaf reaction after 8 days	Symptoms in leaves below growing point after 22 days
31-45 (avirulent)	None apparent	Severe mottling and distortion	25* Small necrotic spots in ill-defined rings	Severe necrosis, mottle, and distortion
50-57 (avirulent)	Slight interveinal yellowing	Light mottle and some distortion	96* Small necrotic spots in ill-defined rings	Some distortion and necrosis
50-58 (avirulent)	Slight interveinal yellowing	Severe mottling and distortion	19* Small necrotic spots in ill-defined rings	Severe necrosis, mottle, and distortion
25-144 (virulent)	Interveinal yellowing and vein banding	Very severe mottling and distortion	90* Spreading necrotic spots covering most of the leaves	Very severe necrosis, mottle, and distortion

* Mean from 3 leaves.

† Variety from Professor C. M. Haenseler, Rutgers University, New Jersey, U.S.A.

V. SPONTANEOUS MUTATION OF AVIRULENT STRAINS OF VIRUS X TO VIRULENT IN *DATURA STRAMONIUM* L.

Following their separation from the virus X complex in the Brownell potato through the use of seedling potatoes (Hutton 1948), the X strains described in this paper were cultured continuously in *D. stramonium* for over two years. During the first year of this period the distinctive properties of the virulent

and three avirulent strains were confirmed a number of times with globe amaranth. However, it became obvious that, in contrast to the virulent strain, the avirulent strains were unstable and mutated to a severe form from time to time in one or two, and sometimes three, of the six replicates of *D. stramonium* used at a serial transfer of each strain. It was thought at first that the appearance of the severe strains in the cultures of the avirulent strains represented an initial admixture of a severe type. The fact that they occurred occasionally and discontinuously during relatively long periods of serial transfer, and then only in one or two replicates of *D. stramonium* at a transfer, disproved this and was strong evidence in favour of mutation.

The mode of development of the severe types in the avirulent cultures was interesting and was additional evidence for their occurrence by mutation. When the instability of the avirulent strains was suspected, close observation of the recently inoculated *D. stramonium* plants after a serial transfer of these strains often revealed one or two small yellow flecks close together in otherwise symptomless leaves of certain plants. When these yellow flecks were excised and inoculated to *D. stramonium* a severe yellow mottle resulted, whereas tissue taken from near the flecks did not produce symptoms in *D. stramonium*, cross-protection tests subsequently proving the avirulent strain to be present. It is apparent from these results that the virulent mutant arises spontaneously in one sector of a leaf, possibly from a single virus particle or aggregate, and owing to its greater biochemical activity is able to compete successfully with the parent avirulent strain so that a small but visible zone of multiplication results. There is no evidence to suggest that virulent mutants arise simultaneously at several separated loci on the same leaf.

It is certain that during the first year of serial transfer of the avirulent strains the number of mutants observed was in excess of the actual number developing, owing to the failure to recognize the first occurrence of a mutant in a leaf. Where a composite leaf sample carrying the avirulent strain contained one leaf with a small unrecognized mutant zone and was used in a serial transfer, the number of apparent mutants developing subsequently would be increased out of proportion. It has been found, using single *D. stramonium* plants, that it usually requires three serial transfers from a leaf carrying a single small yellow fleck to establish finally the virulent mutant in high concentration and suppress the avirulent parent strain. This is illustrated in Plate 2. In Plate 2, Figure 1, on the leaf carrying the avirulent strain 50-58, the light yellow fleck containing the mutant cannot be distinguished. Plate 2, Figure 2, illustrates the results after two serial transfers from the leaf in Figure 1, and Figure 3 illustrates the result of the transfer from Figure 2. When the virulent mutant had become established to the extent shown in Plate 2, Figure 2, it was difficult, although not impossible, to excise tissue giving only the avirulent strain.

During the first year of serial transfers of the avirulent strains the fact that spontaneous mutation occurred was established and the mode of development of the mutants observed. It was evident that little notice could be taken of the rates of mutation of the different strains in this period. In the next 12-

months period a more accurate assessment of the mutation rate of the strains was made by means of serial transfers from a number of separate *D. stramonium* lines of each strain.

The *D. stramonium* lines for this experiment were obtained by inoculating each avirulent strain to batches of 24 young plants and then reinoculating these with a severe strain 21 days later. In both 31-45 and 50-58, eight of the lines failed in this cross-protection test, 50-57, the strain previously shown to give the highest virus concentration in *D. stramonium* with globe amaranth, maintained protection in all 24 plants. Serial transfers were then made from the surviving lines at fortnightly intervals to young duplicate *D. stramonium* plants in the cotyledon stage. Although close observation was made of each line for the occurrence of mutations, a line was not regarded as having produced a spontaneous mutation until yellow flecking was clearly evident. The lines in which mutations developed were discarded. In this way a reasonably accurate comparison of the rates of mutation among the three avirulent strains could be made. The results are summarized in the graph by plotting the number of lines of each avirulent X strain that had not mutated at consecutive monthly periods over the year from March 1949 to March 1950. Thus in the graph the first six months represent the autumn-winter period and the last six months the spring-summer period.

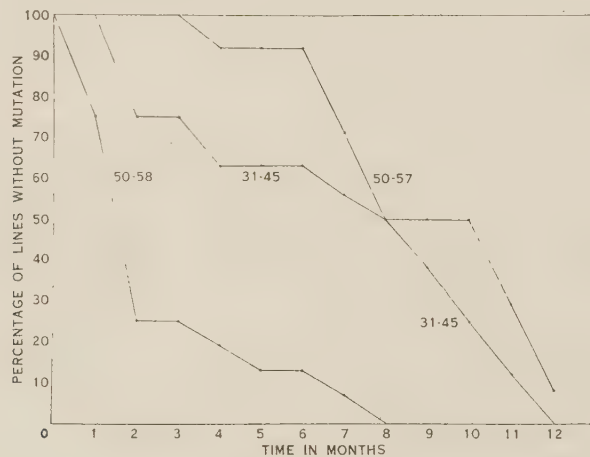


Fig. 1.—Rate of mutation in three avirulent strains of virus X. Strain 50-58 commenced mutating at the beginning of the experiment while 31-45 and 50-57 commenced mutating after one and three months respectively.

In Figure 1 the different mutation rates of the three avirulent X strains can be seen clearly. Strain 50-58 was the least stable, all of its lines being discarded because of mutation in eight months. Strain 50-57, which had been shown previously to develop the highest virus concentration of the avirulent

types in *D. stramonium*, had the lowest mutation rate with two of its lines remaining stable after 12 months. Strain 31-45 had a mutation rate intermediate between 50-58 and 50-57, all lines being finally discarded in 12 months. It is of interest to note that strains 31-45 and 50-58, which developed the lowest concentration in *D. stramonium*, as shown by globe amaranth and *Capsicum frutescens*, had higher mutation rates than 50-57. This suggests that low virus concentrations tend to favour a high mutation rate. Another point of interest in the graph is the suggestion that the increasing photoperiod and rising temperatures of spring and early summer have influenced the mutation rates of the more stable 31-45 and 50-57 strains.

Four times during the course of these experiments *D. stramonium* plants in which mutants from each avirulent strain had developed to the stage shown in Plate 2, Figure 3, were inoculated in properly designed experiments to globe amaranth in comparison with the virulent strain 25-144. No significant differences in virus concentration between the mutants and the virulent strain 25-144 were observed. In addition, there were no real differences between their symptom pictures in *D. stramonium*. It is apparent that when a virulent mutant arises in a culture of an avirulent strain, the mutant, owing to its greater biochemical vigour, eventually suppresses the parent strain to such an extent that it has little or no effect on the virus concentration of the mutant. In addition the mutants observed are indistinguishable from ordinary virulent strains, the course of mutation following the same pattern irrespective of the parent avirulent strain.

VI. DISCUSSION

The mutability of plant viruses is now accepted generally (Bawden 1950) although critical data on the process is rather scanty. This is due in part to the difficulties associated with obtaining strain cultures of many of the plant viruses in a pure condition. Some of the most conclusive early work on the occurrence of mutations in plant viruses was that of McKinney (1935) with tobacco mosaic, while more recently Matthews (1949) demonstrated the mutation of mottle strains of X. From a biochemical standpoint Stanley (1943) considered that the mutation of plant viruses involved changes inducing differences in the proportions of amino acids in the molecules. Bawden (1950) considers that a quantitative change in amino acids is not involved but rather a rearrangement in the sequence of the amino acids on a peptide chain. This is more in line with the type of results obtained by Wyss *et al.* (1948) for induced mutations in bacteria. It will be of considerable interest if it can be shown that the mutations induced in bacteria, fungi, and higher plants are similar in character to those observed in viruses. The work of Gowen (1939) with X-ray-induced mutations in tobacco mosaic, if substantiated by other workers, could be an important link in establishing these relationships.

The work described in this paper gives some indication of the factors influencing spontaneous mutation in avirulent strains of potato virus X. Cultures of these strains maintained in *D. stramonium* without contamination from viru-

lent mutants did not change their relationship to each other as shown by frequent quantitative experiments on globe amaranth during a two-year period. Thus there was no evidence of a back mutation as suggested by Matthews (1949) for his strains. All the evidence presented in this paper indicates that for the avirulent strains of X described, the mutational process is in the direction of increasing virulence as judged by virus concentration and symptomatology in *D. stramonium*. It would be of interest to know whether this type of mutation is usual for mild or avirulent strains of plant viruses, and also whether back mutations occur or could be induced in virulent strains. It is possible that in evolution avirulent strains precede virulent and that avirulent strains could thus remain unnoticed in a host until transferred to a plant favourable to their mutation.

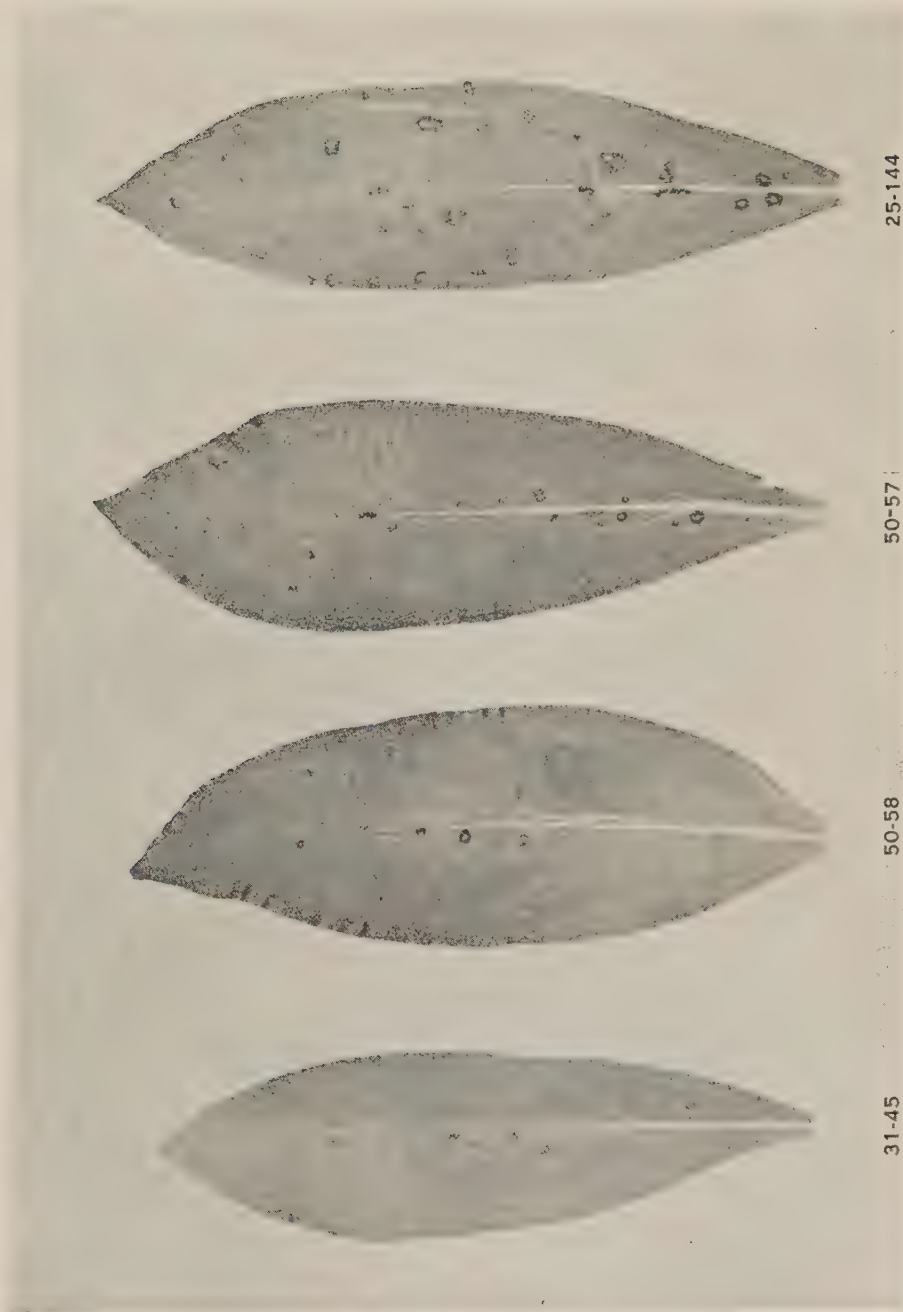
VII. ACKNOWLEDGMENTS

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VIII. REFERENCES

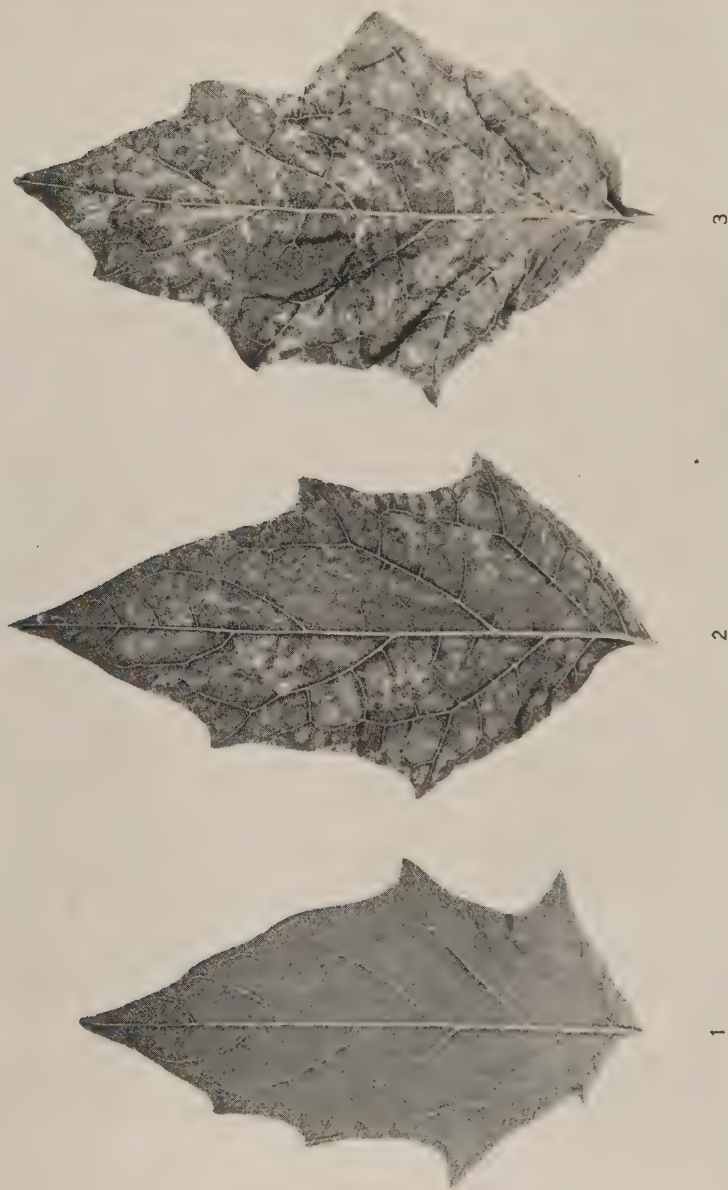
- BAWDEN, F. C. (1950).—"Plant Viruses and Virus Diseases." 3rd Ed. (Chronica Botanica Co.: Waltham, Mass., U.S.A.)
- GOWEN, J. W. (1939).—Radiation effects on biological substances including genes and viruses. Iowa Agric. Exp. Sta. Rep. Part 1, pp. 160-2.
- HUTTON, E. M. (1948).—The separation of strains from a virus X complex by passage through potato seedlings. *Aust. J. Sci. Res. B* 1: 439-51.
- MATTHEWS, R. E. F. (1949).—Studies on potato virus X. I. Types of change in potato virus X infections. *Ann. Appl. Biol.* 36: 448-59.
- McKINNEY, H. H. (1935).—Evidence of virus mutation in the common mosaic of tobacco. *J. Agric. Res.* 51: 951-81.
- SALAMAN, R. N. (1938).—The potato virus "X"; its strains and reactions. *Philos. Trans. B* 229: 137-217.
- STADLER, L. J. (1928).—Mutations in barley induced by X-rays and radium. *Science* 68: 186-7.
- STANLEY, W. M. (1943).—"Virus Diseases." (Cornell University Press: Ithaca, U.S.A.)
- WILKINSON, R. E., and BLODGETT, F. M. (1948).—*Gomphrena globosa*, a useful plant for qualitative and quantitative work with potato virus X. *Phytopathology* 38: Abstr. p. 28.
- WYSS, O., CLARK, J. B., HAAS, F., and STONE, W. S. (1948).—The role of peroxide in the biological effects of irradiated broth. *J. Bact.* 56: 51-7.

MUTATION OF VIRUS X STRAINS



Leaves of globe amaranth showing numbers and types of lesions produced by avirulent X strains 31-45, 50-58, and 50-57 and virulent 25-144.

MUTATION OF VIRUS X STRAINS



D. stramonium leaves illustrating the development of a mutant virulent X strain from the avirulent 50-58.

CHEMICAL INVESTIGATION OF "TRASHY" LEAF PHENOMENON IN AUSTRALIAN-GROWN FLUE-CURED TOBACCO

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Summary

Carbohydrates and nitrogen distribution in flue-cured tobacco leaves described as "trashy" were determined. Sugars in "trashy" leaf were as low as 3 per cent. and in "good" leaf as high as 28 per cent. "Trashy" leaf, relatively to normal leaf, had large total N, protein N, and ammonia N contents and low amide N values, while its weight per unit area was about 30 to 50 per cent. less than that of "good" leaf. Uncured "affected" leaf also had low sugar and high N values and low weight per unit area.

It is believed that trashiness is due to processes associated with carbohydrate impoverishment in leaves on the plant and that its development is conditioned by constraints such as soil nitrogen (N), temperature (T), and sunlight (L). It is suggested that effects of the N - L - T constraints on compounds available to the plant for degradation as "foods," can be expressed in simplified form as $\Sigma R \propto NT$, where ΣR is the total energy "spent" irreversibly (i.e. become non-available for various living processes) by the plant in unit time. If ΣE is the total available energy from all sources during the same time, then for certain values of $\Sigma E - \Sigma R$ "sugars" will decrease with corresponding approach to the state of exhaustion of other compounds and onset of trashiness.

In agricultural practice it is suggested that, when considering measures for control of trashiness, attention should be given to plant spacing, nitrogen supply, number of hours of sunshine, and night temperatures.

I. INTRODUCTION

The phenomenon of tobacco leaves becoming worthless brown material during the flue-curing process occurs every year in tobacco-growing areas of the eastern States of Australia. Such leaves are commonly described as trashy. Loss to the tobacco industry through the occurrence of trashy leaf may be considerable,† the amount varying from season to season and in extreme cases including almost the entire crop in some areas.

The mode of occurrence of this trouble clearly indicates that trashiness is not due to diseases, insect pests, or faulty procedure in harvesting and curing.

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† The season 1932-33 in north Queensland became known as the "black" year. Whole crops were worthless. The economic difficulties of growers were so great that a public inquiry was held and a report (Tobacco Inquiry Committee 1933) presented to the Commonwealth Government. The accumulated evidence showed that the high percentage of trashy leaf in the crop was the major factor affecting production adversely. At that time, tobacco in north Queensland was grown entirely under natural rainfall conditions whereas now a high proportion of the crops are grown under irrigation in the dry season, but trashiness still occurs.

It is developed in the leaf as a result of the conditions under which the leaf was produced, and is a manifestation of intrinsic properties that become more evident during flue-curing. This disposition to trashiness establishes the desirability of obtaining an intimate understanding of the phenomenon and of its relation to known metabolic processes in plants. As far as can be ascertained, no reports on the chemical composition, cause, or occurrence of trashy leaf have been published. Some aspects of the chemical nature of trashy leaf were investigated, special attention being given to nitrogen and carbohydrate distributions.

II. SAMPLING

The importance of sampling of plant material for chemical studies has been emphasized by many chemists (e.g. Chibnall 1924; Smirnov and Izvoschikov 1930; and others) and discussed extensively by Vickery, Leavenworth, and Bliss (1949). Investigations by Vladescu (1938*a*, 1938*b*, 1938*c*) and Andreadis and Toole (1939) on individual leaves of several varieties of tobacco; Piatnitzky (1927) and Darkis *et al.* (1936) on groups of leaves; and Askew *et al.* (1948) on consecutive harvests, all showed that in normally developed plants there is a small change in composition from leaf to leaf depending on stalk position.

To ensure comparative samples for this work, particular attention was given to leaf position on the stalk and to leaf maturity. The mature leaves were harvested and cured or dried. Trashy and good leaves were obtained from the leaf groups represented by leaf positions 4 to 10 inclusive, from the lower half of the plant. All leaves of this group from all the plants in the plot were in seven groups representative of their leaf positions. Each group was then subdivided into three classes—trashy, semi-trashy, and good. Composite samples of each class were obtained by aggregation of proportionate numbers of leaves from each of the seven groups. This procedure reduced errors due to differences in developmental age, should such have been present prior to onset of "trashiness," and ensured comparability of samples throughout the range of leaf positions.

Ripe leaves were harvested in the morning when the amount of carbohydrates is at the lowest level (Moroz-Morozenko 1935) and when differences in sugar contents due to uneven shading through the day are lowest. The cured or dried and graded leaves were stripped, and both web and midrib were dried at 70°C. in an oven with forced air circulation, then ground to pass through No. 36 mesh (openings 0.0170 in.) sieves, and conditioned to contain about 5 per cent. moisture.

*Samples.**—Tobacco plants were of the flue-cured variety, known locally as Kelly (*N. tabacum*), and were grown in plots on the Tobacco Experiment

* Most of the analyses of plant materials have been done on samples obtained during growth and senescence but with flue-cured tobacco the important part of the plant, the leaf, is removed at maturity and subjected to the flue-curing process. Particular attention has therefore been given to the flue-cured leaf, which is the product of growth as modified by curing. The nature of these modifications is well known but further confirmation, under the conditions of the experiments, was obtained by examination of material both before and after curing.

Station of the Queensland Department of Agriculture and Stock at Clare, north Queensland.

The samples 1*d*, 2*w*, 3*n*, 4*n*, and 5*n* are from leaf harvested in November 1948, from plots (each 30 plants) that received uniform cultural treatment in all respects except in quantity of water applied by irrigation. Samples harvested in November 1949, came from the same plot area as those of 1948 but the plants followed soon after a peanut crop and probably had extra N from the soil.

Sample 1*d* was obtained from tobacco plants grown under extremely dry conditions. The plants were stunted (2 ft. high—see Plate 1) and produced comparatively small leaves approximately 18 × 10 in. that failed to ripen normally and retained their green colour after curing.

Sample 2*w* was from plants that always had more than sufficient water, the plants (4 ft. high, topped—see Plate 1) being normally developed with leaves approximately 21 × 12 in. that cured well and were of good quality and colour. Plant development was such that all leaves were exposed to direct sunlight during growth.

Samples 3*n*, 4*n*, and 5*n* were obtained from a plot which received what appeared to be the optimum watering as judged by the appearance of the plants, which were large (6 ft. high, topped—see Plate 1) and had more than the average number of leaves, all of which were of good size, being approximately 24 × 16 in. In the field there was considerable shading of the lower part of the plant and some leaves were thin and lacked gumminess, but these characteristics were not sufficiently pronounced to be used as a basis for the separation of good and trashy leaves. During curing, however, trashiness manifested itself clearly.* Some leaves from these plants were of exceptionally good quality, while others were worthless. From this tobacco three grades were selected: "all trash" (sample 3*n*); thin, papery leaves with brown areas, which may be regarded as "semi-trashy" (sample 4*n*); and high-quality leaf (sample 5*n*).

Samples from the 1949 crop consisted of uncured and cured leaf, which was harvested from alternate plants, the uncured leaf, as harvested, being immediately dried at 70-75°C. Uncured leaf from the same positions on the plants was separated into two groups, representative of "good" and "affected" leaves had they been cured. However, the differences in physical characteristics were small, the leaves with patches from dull green to a brown tinge and somewhat thin being taken to be affected leaf.

Flue-cured samples of 1949 crop were graded into three classes, good leaf, semi-trash, and trash, by the method used for the 1948 samples.

* Affected leaves behave normally during the early stages of the colouring process, but later become brown, whereas normal leaves remain yellow. At the completion of curing, trashy leaves are extremely thin, brittle, and dark brown. These leaves are often referred to as "dead" or "perished." They will not absorb moisture readily and shatter during handling. Trashy leaves after curing weigh only 30-50 per cent. of normal leaves of the same area, a factor of importance because it means that the actual loss of yield is much greater than is apparent on a weight basis. Furthermore, leaves from the lower half of the plant are most commonly affected and such leaves are normally expected to be of good quality and of high value.

III. EXPERIMENTAL

Some chemists have reported their data on tobacco constituents on a dry weight basis and others on weight per unit area (e.g. Smirnov and Izvoschikov 1930; Moroz-Morozenko 1935; and others) the former procedure being considered preferable in this work. However, approximate transpositions to a weight per unit area basis can be made on the basis of comparative weights of equal areas of trashy and good leaves from the same leaf groups. In this connection it must be remembered that the proportion by weight of midrib to leaf-web markedly increases with increasing trashiness. Trashy leaves weighed less per unit area than good leaves and the loss of weight can be substantial. Thus 65 good leaves weighed 605 g. whereas 80 trashy leaves weighed only 242 g., a loss of 67 per cent. in weight for equal areas of leaf. Accordingly this loss of weight per unit area of trashy leaf is taken into account in the interpretation of the results of analyses.

(a) Protein Preparations

These were prepared by removing non-protein nitrogenous compounds, together with non-nitrogenous extractable substances using the method of Lugg (1939) as modified by Lugg and Weller (1944).

(b) Nitrogen

Estimation of total N was made by the Kjeldahl method (Johanson 1948a), using apparatus as described previously (Johanson 1948b, 1949).

(c) Sugars and Starch

The estimation of reducing sugars (glucose and fructose) and sucrose was carried out on the aqueous alcoholic extracts from protein preparations. The aliquots were diluted with water and alcohol was driven off by heating on the water bath. The aqueous extracts obtained were then purified with clearing solution, as described by Doak (1939), and the reducing sugars determined by the procedure of Blick (personal communication; see also Blick 1943; Lepper 1945) by oxidation with Fehling solution, using methylene blue as an indicator. The values for sucrose were obtained by estimating the increase in reducing sugar values after acid hydrolysis. Starch was determined by the method used by Pucher, Leavenworth, and Vickery (1948).

(d) Mineral Matter

The total ash content and soluble and insoluble silica were determined (Askew 1932) and Mg and Ca were estimated by standard methods adopted by the Plant Analysts Committee, New Zealand Institute of Chemistry.

(e) Ammonia

The interference by alkaloids in titrimetric and to a lesser degree in colorimetric estimations of NH_3 N (see also Vickery and Pucher 1929), together with the complicating effect of remaining enzymic activity in tobacco leaf even after

flue curing, made it obligatory to determine the most suitable method for estimation of NH_3 in tobacco. A more detailed report of this investigation will be published elsewhere. The procedure adopted was as follows: Before use, the apparatus (see Pucher, Vickery, and Leavenworth 1935; Archibald 1943) is thoroughly aerated with air scrubbed with H_2SO_4 . The 100 ml. receiver flask is charged with 2 ml. 1N H_2SO_4 and 10 ml. water. Then 2-4 g. of dry sample are placed in the 250 ml. distilling flask together with 20 ml. of 0.25 molar Na_2HPO_4 buffer solution and 10 ml. of water. The distillation is continued for 22 minutes at the reduced pressure of 5-6 cm. Hg, at a temperature of 40-41°C., and a steady stream of air is maintained during the distillation. As soon as the initial frothing subsides, another 20 ml. of water is added through the thistle funnel. The distillate is evaporated to 40 ml. volume in a 100 ml. beaker with 1 ml. of 10 per cent. silicotungstic acid solution, cooled, and set aside for three hours. The solution is filtered into a 100 ml. standard flask using washed No. 42 Whatman filter papers, and the precipitate is washed with 20 ml. N/70 H_2SO_4 . To the filtrate 2 ml. of 1N NaOH is added and 3 ml. of Nessler reagent (Folin 1925; see description by Lepper 1945), diluted to the mark, and allowed to stand for 15 minutes. The colour that develops is then estimated in a photoelectric colorimeter (Hilger) and the concentration of NH_3 N is directly obtained with the aid of a prepared calibration curve.

(f) *Amide N*

The usual methods of estimating amide N are based on the relative instability of amide linkages in an acid medium, the amides being hydrolysed and the liberated NH_3 estimated in the usual manner (see e.g. Borsook and Dubnoff 1939; Pucher, Vickery, and Leavenworth 1935; Lugg 1938; and others). In a careful study Shore, Wilson, and Stueck (1936) presented a reasonably clear picture of the formation of NH_3 during the period of acid hydrolysis, showing that the deamidation reaction is accompanied by the slow deamination of peptides and amino acids. It would appear that, strictly speaking, an exact value requires the measurement of the NH_3 produced after consecutive periods of hydrolysis, and "extrapolation" from the curves thus obtained eliminates NH_3 due to the deamination reactions. However, since the deamination error is very small it is scarcely worth while to correct for it when only comparative values are of interest. After several trials the following procedure was adopted: 1-2 g. dry samples were heated with 50 ml. 1N H_2SO_4 for four hours at 100°C. in 250 ml. flasks, made neutral to bromophenol blue with 5N NaOH, and after addition of 20 ml. of 0.25M Na_2HPO_4 buffer the procedure followed that described for the estimation of preformed ammonia.

(g) *Nicotines*

The estimation of nicotine alkaloids in tobacco has been extensively considered by many workers (e.g. Bowen and Barthel 1944; Markwood and Barthel 1943; Bowen 1947). It would appear that steam distillation, with subsequent precipitation of the alkaloids (notably nicotine and *nornicotine*) with silico-

tungstic acid, offers the simplest and most accurate means for their determination. While it is necessary to recognize that the values obtained by such methods are somewhat arbitrary, depending on nicotine-*nor*nicotine ratios and amounts of minor steam-volatile alkaloids (nicotyrine, nicotimine, and anabasine; see Smirnov 1940), the total nictines within the same variety of tobacco from the same area may be taken, for comparative purposes, as representative of their alkaloid contents.

TABLE 1
NITROGEN IN TOBACCO LEAF-WEB SAMPLES AND IN THEIR PROTEIN PREPARATIONS

Samples*	Fresh wt. Taken (g.)	Moisture (%)	Dry Wt. (g.)	Total N (%)	N Total (mg.)	Coagulable N (mg.)	Protein N (mg.)	N in "Protein" Prep. (%)	Dry Wt. "Protein" Prep. (g.)
1 <i>d</i> Still green after curing	80.7	5.24	76.5	3.41 1.89†	2608	1525	1500	3.63	41.4
2 <i>w</i> Good leaf	80.0	7.29	74.2	1.64 1.39†	1218	637	627	1.94	32.3
3 <i>n</i> All trash	60.0	6.04	56.4	1.83 1.62†	1031	587	582	1.57	37.2
4 <i>n</i> Semi- trash	75.0	4.55	71.6	1.82 1.58†	1301	688	682	1.73	39.4
5 <i>n</i> Good leaf	85.0	4.77	80.9	1.81 1.43†	1462	733	724	1.88	38.6
‡Good leaf (uncured)	47.6	7.36	44.1	2.05	906	645	636	2.70	23.6
‡Affected (uncured)	45.3	5.14	43.0	2.25	966	702	697	2.91	24.0

* Grown under — "*d*"—dry, "*w*"—wet, and "*n*"—normal watering conditions.

† Total N in midrib of the corresponding samples.

‡ From 1949 crop; all others 1948 crop.

Procedure.—Samples of 1-2 g. were distilled with 10 ml. 8N NaOH, 5 g. NaCl, and 100 ml. of water the volume of which was maintained, under reduced pressure of about 7 cm. Hg at 90°C. in the apparatus used for the determination of preformed ammonia. The distillate was collected in 20 ml. of 0.5N HCl until no opalescence appeared when some of it was treated with a drop of silicotungstic acid. The distillate was evaporated to 200 ml. with 5 ml. of 10 per cent. silicotungstic acid ($4\text{H}_2\text{O} \cdot \text{SiO}_2 \cdot 12\text{WO}_3 \cdot 22\text{H}_2\text{O}$) and allowed to stand overnight. The crystalline precipitate was filtered through No. 42 Whatman papers and washed with N/70 HCl until free from silicotungstic acid. The papers were folded, dried in Pt crucibles to drive off the remaining HCl, and then strongly ignited to constant weight. Weight of residue $\times 0.1141$ was taken as the weight of nictines. Individual determinations agree within less than 1 per cent.

IV. RESULTS

The reported values in Tables 1, 2, 3, and 4 are the means of closely agreeing duplicate or replicate estimations. In Table 1 are shown the percentages of total N, N in "protein" preparations, the amounts of total N, coagulable N, protein N, and other data concerning the preparations. The variation trend of total N in the tobaccos from the 1948 crop is clearly affected by the conditions of growth. The total N and protein N (see Table 2) in 1*d* are greater than any values recorded elsewhere for flue-cured tobaccos (see e.g. Frankenburg 1946) and are correlated with extremely poor smoking quality. The total N content is least in 2*w* whilst in 3*n*, 4*n*, and 5*n* the total N values are similar when expressed on a dry weight basis. It is also evident that the N values of the 1948 crop are much lower than those for 1949 crop (Table 4).

TABLE 2
NITROGEN DISTRIBUTION IN CURED TOBACCO LEAF-WEB SAMPLES, 1948 CROP

Samples	Protein N in Dry Sample (%)	Total Ex- tractables (%)	Ratio Ex- tractables/Pro- tein N	Total N (%)	NH ₃ N (% × 100)	NH ₃ N of Total N (%)	Amide N (%)	Amide N of Total N (%)	Nicotines (%)
1 <i>d</i> Still green after curing	1.97	45.9	23.4	3.41	3.20	0.94	0.316	9.27	3.11
2 <i>w</i> Good leaf	0.84	56.5	67.0	1.64	0.021	0.013	0.128	7.80	0.86
3 <i>n</i> All trash	1.03	34.1	33.0	1.83	0.104	0.57	0.085	4.64	1.72
4 <i>n</i> Semi- trash	0.95	44.9	47.1	1.82	0.050	0.028	0.130	7.15	1.65
5 <i>n</i> Good leaf	0.89	52.4	58.6	1.81	0.020	0.011	0.155	8.58	1.77

In Table 2 the contents of nicotines, NH₃ N, and amide N are given, and the last two are expressed as percentages of total N; the values of protein N and total extractables are expressed as a percentage of dry samples. Incidentally, the criterion for inclusion of substances in the class of extractables (comprising compounds of three major groups, viz. static, dynamic, and non-protein nitrogenous—see Frankenburg (1946)) is based entirely on their property of remaining in plasmolysing and aqueous alcoholic solvents during the preparation of "proteins." From the N distribution of samples 3*n*, 4*n*, and 5*n* it is apparent that, chemically, trashy leaf is not "dead" in the usually accepted sense because total N and protein N (see Table 2) when expressed on a dry weight basis are normal. This trend is also apparent in total N (1.39 to 1.62 per cent.) in the corresponding midrib samples (Table 1). The development of trashiness is shown even more clearly by the notable decrease of the percentage total extractables (viz. from 52.4 to 34.1 per cent.) also by the ratio

of extractables to protein N. Similar trends are evident in leaf from the 1949 crop (Table 4) where percentages of N increase with trashiness in both cured and uncured leaf.

It must be noted, however, that the increase in N values is only relative to other constituents. Had the data been expressed on a weight per unit area basis the absolute amount of N would have shown a marked decrease. A much greater rate of decrease in other constituents, mainly carbohydrates, produces the effect of an apparent increase in N. Furthermore, these changes in constituents are of such magnitudes that variations due to leaf position (Vladescu 1938*a*, 1938*b*, 1938*c*), within the range selected, become unimportant.

The amounts of NH_3 N (Table 2) are much smaller in the cured leaf than those reported by other workers (e.g. Vickery *et al.* 1940) for the fresh tobacco leaf. Some loss of ammonia, depending on pH of the tissues, must be expected during curing since a temperature of 85°C . is often attained. However, a definite trend to higher NH_3 contents with the progress of trashiness is apparent. The amide N values in samples 3*n*, 4*n*, and 5*n* clearly indicate that there is progressively less of this nitrogen as the degree of trashiness increases. Possibly a greater proportion of amide N is formed in good leaf than in trashy leaf during curing, depending on relative amounts of carbohydrates present. Such amide formations in detached leaf would be in accordance with reports made in the literature (see e.g. Vickery *et al.* 1937; Chibnall 1939; Street 1949). The value of nictines appears to vary mainly with respect to water treatments and to be less per unit area in trashy than in the good leaf.

TABLE 3
SUGARS AND MINERAL MATTER IN CURED TOBACCO LEAF-WEB, 1948 CROP

Samples	Starch as Glucose (%)	Reducing Sugars (%)	Sucrose (%)	Total "Sugars" (%)	Non-Sugar Extractables (%)	Ca (%)	Mg (%)	Soluble Silica (%)	Insoluble Silica (%)	True Ash (%)
1 <i>d</i> Still green after curing	—	3.59	1.64	5.31	40.7	2.54	0.60	0.54	1.39	13.4
2 <i>w</i> Good leaf	1.71	24.4	3.62	28.2	28.5	1.90	0.39	0.45	0.93	10.0
3 <i>n</i> All trash	0.00	3.06	0.23	3.30	30.8	—	—	—	—	—
4 <i>n</i> Semi-trash	0.40	11.9	0.32	12.2	32.7	—	—	—	—	—
5 <i>n</i> Good leaf	0.80	19.3	0.44	19.7	32.7	2.17	0.44	0.63	1.30	12.6

In Table 3, percentages of sugars and mineral matter are given. The values for Ca, Mg, true ash, and silica are lowest in sample 2*w* and highest in 1*d* except for the soluble silica. Thus the general distribution of the mineral fractions clearly indicates a trend toward increases in values with drier growth conditions.

Trends in nitrogen are of significance, but the more spectacular chemical indication of trashiness is given by the carbohydrates. Thus, as shown in Table 3 (1949 crop), 28.2 per cent. of total sugars are present in good leaf (2*w*) and only 3.30 per cent. in trashy leaf (3*n*). Sugars may amount to half of the total extractables, depending on the "quality" of the leaf, total sugar contents increasing with decrease in trashiness. The percentages of non-sugar extractables (Table 3) in good and trashy leaf are somewhat similar. This means that in the flue-cured leaf the leading role in the major quantitative changes in the extractables must be conceded to the sugars which, of all the carbohydrates, are the major constituents of the dynamic group (see Frankenburg 1946).

The above trends in sugars are also present in the 1949 crop, in both cured and uncured leaf samples (Table 4). Total sugars in cured leaf (expressed as glucose) drop from 23 per cent. in "good" leaf to 2.5 per cent. in trashy leaf, while the uncured "affected" leaf has 8 per cent. less total sugars than its counterpart uncured "good" leaf.

V. DISCUSSION

When the data in the previous sections are considered in conjunction with the extensive evidence available in the literature, and with observations of conditions under which tobacco is grown, they suggest the probable causes of trashy leaf. In evaluating the causes, the effects, if any, of flue-curing have to be taken into account. Results of extensive investigations (e.g. Frankenburg 1946) show that total changes are such that percentage compositions of a leaf before and after curing are somewhat similar and the total loss of dry matter is of the order of 10 per cent. Thus it has been shown that the final composition of the flue-cured leaf is dependent on the initial composition of the green leaf, therefore trashiness does not develop as a result of flue curing.

The results of chemical analysis (Table 4) of cured and uncured leaf of both groups (trashy and "good" leaf) proved beyond doubt that the identification of affected leaf before curing was possible. Physical characteristics used in 1949 as a basis for separation of uncured leaf into "affected" and "normal" leaf were observed in 1950 in the field on plants that produced trashy leaf, thus confirming the association of trashiness with conditions of growth. These findings indicate that affected uncured leaf (or at least most of it) is lower in sugars, has high N content, and has less weight per unit area than normal uncured leaf. Consequently, it must be concluded that trashiness is due to intrinsic properties of affected uncured leaf and is not produced by the flue-curing process. These intrinsic properties, whatever they are, are associated with low carbohydrate and high N contents on a dry weight basis or weight per unit area basis.

Incidentally, such a conclusion would explain, in part, the higher ammonium N and lower amide N contents in trashy leaf, as these substances are likely to form in such proportions to one another during the starvation period of curing in a leaf low in carbohydrate.

As it has been shown that uncured affected leaf is low in "sugars" and high in N, it is reasonable to assume that processes during growth and ripening of the leaf are responsible for this occurrence. From observations made in tobacco fields during the past 20 years, it appears that trashy leaf comes from areas where the soil is high in N and where night temperatures are relatively high, and particularly from shaded portions of plants in high crops. Occasionally cloudiness may persist over a wide area for relatively long periods, thus simulating shaded conditions within a crop. In the disastrous 1932-33 season in north Queensland, persistent clouds and light rain for a period of three weeks when the crops were about to mature provided extremely favourable conditions for the development of trashiness. In 1950, trashiness developed under similar conditions and persisted until rain and clouds were replaced by brilliant sunshine.

Evidence has accumulated to show that with tobaccos grown on soils high in N, carbohydrate contents are lower than for tobaccos grown in low N soils and also that high N content is invariably associated with relatively low sugar values. An interesting example of this relationship was reported by Askew *et al.* (1948) where, with a sudden uptake of N from the soil, the general level of 28 per cent. sugar dropped to 16 per cent. while N increased from 1.9 to 3.0 per cent. Similarly, Woltz, Reid, and Colwell (1948) found that sugar content in the cured leaves of flue-cured tobacco was inversely related to total N applied to the soil. In an experiment during 1949, plants (including controls and guard rows) were distinctly deficient in N (as was verified by additions of N to fully grown plants and observations on the radical change that followed the applications), the leaf was smaller and thicker than usual and yellow in colour. Analysis of this leaf revealed exceptionally high sugar content, up to 43 per cent., while N values were of the order of 1 per cent. (expressed on dry weight basis). In general, flue-cured varieties grown on soils excessive in N have leaf high in proteins and other nitrogenous constituents and are very low in sugars. These tobaccos are at best of "poor quality," often will not cure but "decompose," and represent a loss to the tobacco industry.

How N as a constraint through metabolic reactions causes depletion of sugars in the leaf is in the field of speculation and remains to be determined. However, processes removing carbohydrates from the leaf are respiration, formation of organic N compounds, cell wall material, pigments, phenols, essential oils, etc., and translocation from the leaf to other parts of the plant. Therefore the carbohydrate content of a leaf at any time is a measure of the rate of formation of "sugars" (mainly by photosynthesis) and rate of loss. These considerations would explain how environmental conditions conducive to loss of sugars (e.g. such as excessive rate of respiration at high temperature etc.) or interruptions of photosynthesis (e.g. insufficient sunlight etc.) would lead to formation of affected leaf, while retarded consumption of carbohydrates and prolonged photosynthesis would lead to accumulation of sugars.

Since environmental factors, at any one time, would favour either increase or loss of sugars; depending on the sum total of these effects, various contents of carbohydrates in the affected leaf must be expected. Closer examination

of analytical data suggests that in Figures 1 and 2 correlation between sugars and "extractables," and protein N, originate from various degrees of "trashiness." On a dry weight basis the protein N content of trashy leaf is relatively high compared with normal leaf and as "trashiness" progresses, removal of sugars and "labile" compounds (e.g. essential oils, phenols, etc.) produces an apparent increase in N and hence the correlation.

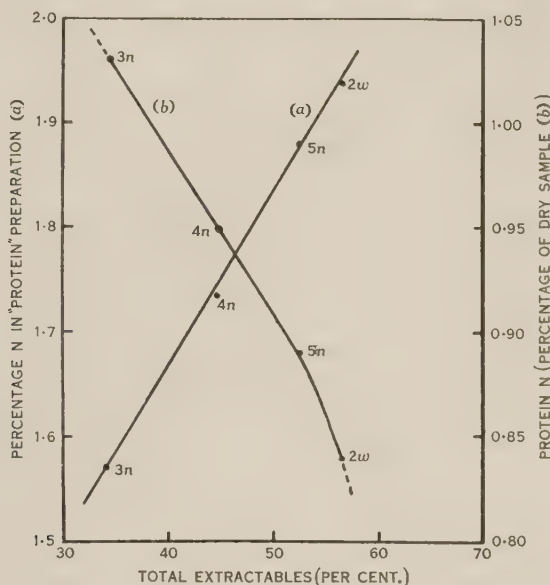


Fig. 1.—Changes in protein N and total extractables with development of "trashiness."

From this, the distribution of 3n, 4n, 5n, and 2w samples about a straight line (Fig. 1, curve (a)) could be interpreted as showing that these widely varying samples come (or would have come) from leaf with somewhat similar composition and affected by somewhat similar environmental constraints. The environmental conditions under which 1d was grown produced "tobacco" of an entirely different nature which, with its extremely high non-protein N and low sugars, could be regarded as a typical product of drought (see also Petrie and Arthur 1943). Further along the line 2w → 5n → 4n trashiness is latent and small adverse changes in environment may produce semi-trashy and trashy leaf, viz. 4n and 3n. This is again apparent from Figure 1, curve (b), with perhaps an additional indication that, as trashiness progresses, the relative rate of decrease of extractables is greater than that of protein N. On the other hand (see Fig. 2) the rate of decrease of total "sugars" with advance of trashiness is somewhat constant in 5n, 4n, and 3n, with the possibility that 2w is still further removed from the tendency to become trashy. The difference of 8.5 per cent. for total sugars, between 2w and 5n, indicates that 5n may lie at the limit where infinite "gradation" is represented by a linear function of the curve.

Weather conditions during 1948 and 1949 were not the same, nevertheless values for N and sugars for the 1949 crop (Table 4) conform to the nitrogen-sugars relationship established for the 1948 crop, high N values being associated with low sugars contents.

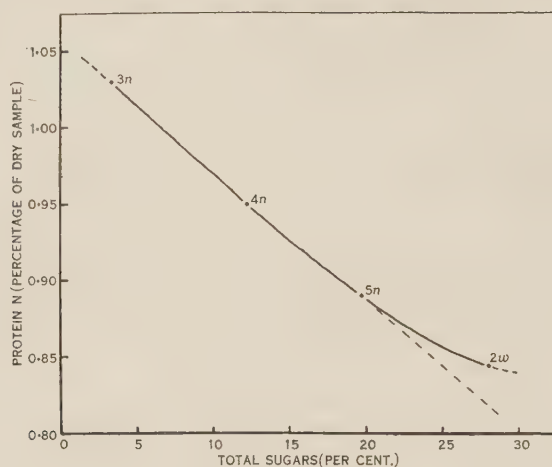


Fig. 2.—Changes in protein N-sugars relationship with development of “trashiness.”

The quantitative chemical changes of constituents in the leaf and field observation of conditions with which trashiness is associated show that the

TABLE 4
NITROGEN AND SUGARS IN CURED AND UNCURED LEAF, 1949 CROP

Samples	Total N (%)	Protein N in Dry Sample (%)	Reducing Sugars (%)	Sucrose (%)	Starch as Glucose (%)	Total Sugars as Glucose (%)
Good leaf (cured)	2.03	1.16	14.9	4.70	3.12	22.7
Semi-trash (cured)	2.38	1.34	8.62	3.81	1.18	13.6
Trash (cured)	2.49	1.49	1.47	0.31	0.76	2.53
Good leaf (uncured)	2.05	1.45	17.4	1.55	5.64	24.6
Affected (uncured)	2.25	1.62	9.24	2.20	5.26	16.7

results of this phenomenon have striking similarities to occurrences in other field crops, e.g. sugar cane, cotton, sugar beet, pineapple, etc. Ulrich (1942)

stressed the importance of sunlight and warm days but cool nights as conditions favouring the storage of sugar by high-nitrogen sugar beets; conditions conducive to rapid synthesis of sugars and limited loss of them by respiration. Gardner and Robertson (1942) in a study of the effect of N fertilizers on beets concluded that over-fertilization with N results in a relatively low percentage of sugar in the storage roots and in excessive leaf growth. Dunlap (1945) concluded that conditions unfavourable for the synthesis of sugars, such as cloudy weather, artificial shade, close spacing of plants, or reduction in the number of hours of sunlight increased abscission of cotton bolls, while Eaton and Rigler (1945) found that shedding of bolls was associated with limited carbohydrate reserve. The extensive work on cotton (e.g. Wadleigh 1944; Dastur and Ahad 1941; Eaton and Rigler 1945; Dunlap 1945) reveals that the supply of N available for protein synthesis may be critical in relation to the carbohydrate contents of plants and conditions favouring the synthesis of sugars. Wadleigh (1944) states that, with high N supply, cotton plants may become too vegetative for optimum boll production, and the lower the N the higher may be the carbohydrate reserve of the plants. It may be noted that carbohydrate reserves were directly related to the formation of cellulose fibres and to the oil content of the cotton seeds. Again, Ulrich (1950) reports that nitrate concentrations of the petioles were correlated inversely with sugar concentrations of beet roots; the higher the nitrate values, the lower the sugar concentrations.

High N supply from the soil possibly stimulates the utilization of carbohydrates but subsequent analysis of a trashy leaf may not indicate high organic nitrogen content owing to later degeneration changes and translocation arising from the low carbohydrates level. Thus with trashiness a limit may be reached in the absence of carbohydrates when not only further synthesis of organic N compounds cannot proceed normally, but overall degradation of stored products, as a source of energy for respiratory processes, may become intensified.

Again, investigations with sugar cane (see Brink and Van Den Honert 1940; Rosenfeld 1937) show that a critical balance exists between supply of N and accumulation of sugar in cane. In environments where sunlight is low, decreased yields of sugar resulted if the N applications were too high (see Borden 1940, 1944). The work of Clements (1940), Clements and Moriguchi (1942), and Clements and Kubota (1943) with sugar cane showed that, in locations where soil moisture and soil nutrients were essentially the same, the marked difference in yields of sugar is correlated with differences in sunlight. It would thus appear from numerous reports (see Nightingale 1948) that variants such as soil nitrogen (*N*), sunlight (*L*), and temperature (*T*) (see also Foster and Tatman 1938; Went 1944, 1945; Gregory and Sen 1937; Nightingale 1942) exert a powerful influence on metabolic processes in plants and that unfavourable combinations of these variants (*N-L-T*) may be detrimental to the production of a crop. The effect of adverse *N-L-T* magnitudes may appear different in unrelated crops, but the operative constraints are the same.

In tobacco, as far as is known of conditions under which affected leaf occurs, the affected plants appear to be normal (relative to those with good leaf) and there is no circumstantial evidence nor suspicion of any trace element deficiencies or toxicity, nor is there evidence of any deficiencies in major inorganic nutrients or in water requirements. Thus, of the possible environmental constraints that would produce affected leaf, the findings of these investigations, when examined in the light of data presented in the literature, lead to the belief that this phenomenon is a direct outcome of unfavourable magnitudes of $N-L-T$. The relative importance of temperature as a major constraint should not be underestimated. In an extensive study on bean, milkweed, and tomato plants, Hewitt and Curtis (1948) found an average of 15 per cent. total loss of dry matter by respiration and translocation in a 13-hour period with increase of temperature from 10° to 30°C., while in the same period the carbohydrate (starch and soluble sugars) content of the bean leaves dropped by 50 per cent. Thus in addition to the effect of N on depletion of "sugars" in the tobacco plant as a whole it must be expected that such depletion would be accelerated by an increase in T , which would also directly affect the rate of respiration and consequently the total available energy from carbohydrates. In addition to this general effect an increase in T accelerates translocation and thus further aggravates the tendency to trashiness in affected leaves already low in "sugars."

Incidentally, the impoverishment of carbohydrates in a leaf, as was found in careful investigations by workers in this field (e.g. Moroz-Morozenko 1935; and others), may be very rapid. Under unfavourable conditions, leaf can become completely devoid of its "sugars" within 24-48 hours, reaching a state of exhaustion.

As already stated, processes responsible for sugar exhaustion in the leaf are respiration, formation of cell wall materials, organic N compounds, essential oils, pigments, etc., and translocation from the leaves to other parts. Some of these processes would make "sugars" (or products of "sugar") partially or totally non-available to carry on life requirements should the fresh supplies from photosynthesis be insufficient or stopped. At the stage when supply of "sugars" is insufficient or exhausted and the plant begins to "starve," other compounds such as pigments, essential oils, phenols, etc., disappear (possibly degraded as "foods") and in tobacco leaf "trashiness" develops.

Since the "sugar" exhaustion and degradation of products has such far-reaching effects, attention is drawn to the associated loss of energy (E). Plants may have all the inorganic salts and elements necessary to build components, yet, in the absence of E , synthesis of "foods," structural and auxiliary units, enzymes, etc. necessary for life processes will not be possible. If E is not stored in readily available compounds for degradation, for use in 'repair jobs,' for removal of mechanical strains, etc., life cannot persist.

Thus a consideration of the E requirements of a plant suggests that $N-L-T$ as constraints, by producing effects on living processes also produce immediate corresponding effects on total *available* E supply for these processes; remembering that increase in N increases demands on carbohydrates, increase in

T increases rate of respiration, and L through photosynthesis is stored in "foods" as chemical energy.

The rapidity with which "sugars" disappear and magnitudes to which they accumulate suggest their important role in available E change in the plant. Let ΣE be the total *available* energy from all sources, including light, during a unit time and ΣR be the total energy "spent" irreversibly* (i.e. become non-available) for the same unit time. Taking S as a value such that $\Sigma E - \Sigma R = S$ when contents of "sugars" and similar compounds in a plant are just adequate, then if $\Sigma E - \Sigma R > S$, "sugars" will increase and if $\Sigma E - \Sigma R < S$, "sugars" will decrease with a corresponding approach to the state of exhaustion in the plant. Further, the effect of N or T variation on ΣR , for small increments when simplified may be written as $\Sigma R \propto NT$. Thus with increase of T , the magnitude of ΣR may be kept somewhat unaltered if N is decreased, thereby tending to maintain the carbohydrate level and ward off trashiness.

From these considerations it is concluded that conditions favourable for suppression of trashy leaf development would be associated with practices that ensure a small ΣR and large ΣE . In agricultural application it would appear that the former could be decreased by providing the minimum necessary nitrogen supply and by producing the crop in areas where the rate of respiration is retarded by relatively low night temperatures, while the latter could be increased by wider spacing of plants and by growing them in areas where days are relatively long. In this connection it may be that in flue-curing areas of North America, where temperatures are somewhat similar to those in north Queensland, the greater number of hours of sunshine is of major importance in maintaining carbohydrates at a level above which trashiness is not apparent.

VI. ACKNOWLEDGMENTS

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VII. REFERENCES

- ANDREADIS, T. B., and TOOLE, E. J. (1939).—*Z. Untersuch. Lebensmitt.* **77**: 262.
 ARCHIBALD, R. M. (1943).—*J. Biol. Chem.* **151**: 141.
 ASKEW, H. O. (1932).—*N.Z. J. Sci. Tech.* **14**: 92.
 ASKEW, H. O., BLICK, R. T. J., CURRIE, K. E., and WATSON, J. (1948).—*N.Z. J. Sci. Tech.* **A 30**: 129.
 BLICK, R. T. J. (1943).—*N.Z. J. Sci. Tech.* **B 25**: 53.

* Here irreversible expenditure of E is associated with processes or that part of processes that result in compounds becoming totally or partly non-available to the plant for degradation as "foods" (e.g. by respiration and formation of cell wall material) while on the other hand formation of essential oils, oligosaccharides, etc. is regarded as a reversible process.

- BORDEN, R. J. (1940).—*Hawaii. Plant. Rec.* **44**: 237.
BORDEN, R. J. (1944).—*Hawaii. Plant. Rec.* **48**: 197.
BORSOOK, H., and DUBNOFF, J. W. (1939).—*J. Biol. Chem.* **131**: 163.
BOWEN, C. V. (1947).—*J. Ass. Off. Agric. Chem.* **30**: 315.
BOWEN, C. V., and BARTHEL, W. F. (1944).—*Industr. Engng. Chem. (Anal. Ed.)* **16**: 377.
BRINK, R., and VAN DEN HONERT, T. H. (1940).—*Sug. News* **21**: 553.
CHIBNALL, A. C. (1924).—*Biochem. J.* **18**: 387.
CHIBNALL, A. C. (1939).—"Protein Metabolism in the Plant." (Yale Univ. Press: New Haven, U.S.A.)
CLEMENTS, H. F. (1940).—*Hawaii. Plant. Rec.* **44**: 201.
CLEMENTS, H. F., and MORIGUCHI, S. (1942).—*Hawaii. Plant. Rec.* **46**: 163.
CLEMENTS, H. F., and KUBOTA, T. (1943).—*Hawaii. Plant. Rec.* **47**: 257.
DARKIS, F. R., DIXON, L. F., WOLF, F. A., and GROSS, P. M. (1936).—*Industr. Engng. Chem. (Anal. Ed.)* **28**: 1214
DASTUR, R. H., and AHAD, A. (1941).—*Indian J. Agric. Sci.* **11**: 279.
DOAK, B. W. (1939).—*N.Z. J. Sci. Tech.* **B 21**: 90.
DUNLAP, A. A. (1945).—*Texas Agric. Exp. Sta. Bull.* No. 677.
EATON, F. M., and RICLER, N. E. (1945).—*Plant Physiol.* **20**: 380.
FOLIN, O. (1925).—"Laboratory Manual of Biological Chemistry." (D. Appleton and Co.: London.)
FOSTER, A. C., and TATMAN, E. C. (1938).—*J. Agric. Res.* **56**: 869.
FRANKENBURG, W. G. (1946).—*Adv. Enzymol.* **6**: 309.
GARDNER, R., and ROBERTSON, D. W. (1942).—*Colo. Sta. Coll. Agric. Exp. Sta. Tech. Bull.* No. 28.
GREGORY, F. G., and SEN, P. K. (1937).—*Ann. Bot. Lond.* **1**: 521.
HEWITT, S. P., and CURTIS, O. F. (1948).—*Amer. J. Bot.* **35**: 746.
JOHANSON, R. (1948a).—*Aust. J. Exp. Biol. Med. Sci.* **26**: 259.
JOHANSON, R. (1948b).—*Aust. Chem. Inst. J. Proc.* **15**: 183.
JOHANSON, R. (1949).—*R. Aust. Chem. Inst. J. Proc.* **16**: 483.
LEPPER, H. A. (1945).—"Methods of Analysis of the Association of Official Agricultural Chemists." 6th Ed. (Ass. Off. Agric. Chem.: Washington.)
LUGG, J. W. H. (1938).—*Biochem. J.* **32**: 2114.
LUGG, J. W. H. (1939).—*Biochem. J.* **33**: 110.
LUGG, J. W. H., and WELLER, R. A. (1944).—*Aust. J. Exp. Biol. Med. Sci.* **22**: 149.
MARKWOOD, L. N., and BARTHEL, W. F. (1943).—*J. Ass. Off. Agric. Chem.* **26**: 280.
MOROZ-MOROZENKO, M. G. (1935).—*State Inst. Tobacco Invest. Bull.* No. 118. Krasnodar, U.S.S.R.
NIGHTINGALE, G. T. (1942).—*Bot. Gaz.* **103**: 409.
NIGHTINGALE, G. T. (1948).—*Bot. Rev.* **14**: 185.
PETRIE, A. H. K., and ARTHUR, J. I. (1943).—*Aust. J. Exp. Biol. Med. Sci.* **21**: 191.
PIATNITZKY, M. (1927).—*State Inst. Tobacco Invest. Bull.* No. 28, Krasnodar, U.S.S.R.
PUCHER, G. W., VICKERY, H. B., and LEAVENWORTH, C. S. (1935).—*Industr. Engng. Chem. (Anal. Ed.)* **7**: 152.
PUCHER, G. W., LEAVENWORTH, C. S., and VICKERY, H. B. (1948).—*Anal. Chem.* **20**: 850.
ROSENFELD, A. H. (1937).—*Min. Agric. Egypt Tech. Sci. Serv. Bull.* No. 169.
SHORE, A., WILSON, H., and STUECK, G. (1936).—*J. Biol. Chem.* **112**: 407.
SMIRNOV, A. I. (1940).—"Biochemie des Tabaks." (Junk: The Hague.)
SMIRNOV, A. I., and IZVOSCHIKOV, W. P. (1930).—*Biochem. Z.* **228**: 329.
STREET, H. E. (1949).—*Adv. Enzymol.* **9**: 410.
TOBACCO INQUIRY COMMITTEE (1933).—*Report.* (Commonw. Govt. Printer: Canberra.)
ULRICH, A. (1942).—*Proc. Amer. Soc. Sug. Beet Tech.* **3**: 66.
ULRICH, A. (1950).—*Soil. Sci.* **69**: 291.
VICKERY, H. B., and PUCHER, G. W. (1929).—*J. Biol. Chem.* **83**: 1.

"TRASHY" LEAF IN AUSTRALIAN TOBACCO



Fig. 1.—Typical tobacco plant grown under dry, "d", conditions.

Fig. 2.—Tobacco plant grown under wet, "w", conditions.

Fig. 3.—Tobacco plant grown under normal, "n", conditions.

- VICKERY, H. B., PUCHER, G. W., WAKEMAN, A. J., and LEAVENWORTH, C. S. (1937).—Conn. Agric. Exp. Sta. Bull. No. 399.
- VICKERY, H. B., PUCHER, G. W., WAKEMAN, A. J., and LEAVENWORTH, C. S. (1940).—Conn. Agric. Exp. Sta. Bull. No. 442.
- VICKERY, H. B., LEAVENWORTH, C. S., and BLISS, C. I. (1949).—*Plant Physiol.* **24**: 335.
- VLADESCU, I. D. (1938*a*).—*Z. Untersuch. Lebensmitt.* **75**: 167 (From *Tabul. Biol., Berl.* **18** (4): Haag 1940).
- VLADESCU, I. D. (1938*b*).—*Z. Untersuch. Lebensmitt.* **75**: 340 .
- VLADESCU, I. D. (1938*c*).—*Z. Untersuch. Lebensmitt.* **75**: 450.
- WADLEIGH, C. H. (1944).—Arkans. Agric. Exp. Sta. Bull. No. 446.
- WENT, F. W. (1944).—*Amer. J. Bot.* **31**: 597
- WENT, F. W. (1945).—*Amer. J. Bot.* **32**: 469.
- WOLTZ, W. G., REID, W. A., and COLWELL, W. E. (1948).—*Soil Sci. Soc. Amer. Proc.* **13**: 385.

STUDIES IN THE METABOLISM OF PLANT CELLS

IX. THE EFFECTS OF 2,4-DINITROPHENOL ON SALT ACCUMULATION AND SALT RESPIRATION

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Summary

2,4-Dinitrophenol, while increasing the respiration, inhibits the accumulation of ions by carrot cells. Further investigation is necessary to determine whether the inhibition is due to a direct effect of the dinitrophenol on the mechanism or whether the dinitrophenol indirectly prevents the mechanism from operating by causing some disorganization within the cell, possibly in the mitochondria. If the assumption that dinitrophenol inhibits phosphate transfers is justifiable, hypotheses of salt accumulation might require modification to allow for the participation of energy-rich phosphate. This would suggest that the Lundegardh mechanism may be a part of a more complex mechanism.

I. INTRODUCTION

During the last ten years, Lundegardh (cf. Lundegardh 1945) has developed a theory for the mechanism of accumulation of ions by plant cells. He suggests that the accumulation mechanism is dependent on the transfer of electrons by the cytochrome system and the simultaneous liberation of hydrogen ions. He thus connects the accumulation mechanism to the respiration. Robertson and Wilkins (1948) have shown that the accumulation rates are not inconsistent with such a hypothesis, and Weeks and Robertson (1950) have shown that the respiration is undoubtedly dependent on cytochrome oxidase.

Suggestions (cf. Hoagland 1944) have been made that the accumulation mechanism may be dependent on phosphorylation, since the only well-understood energy transfer system involves phosphate transfer. Nance (1949), observing that 2,4-dichlorophenoxyacetic acid (2,4-D) inhibited accumulation without affecting respiration, suggested that this may be due to an inhibitory effect of 2,4-D on phosphate transfers, but it has not been proved experimentally that 2,4-D affects phosphate transfers.

Loomis and Lipmann (1948) have suggested that 2,4-dinitrophenol (DNP) replaces phosphate in the oxidation and thus uncouples phosphorylation, while not lowering or even slightly stimulating the rate of oxidation. An alternative explanation of the effect of dinitrophenol on oxidation rate has more recently been suggested by Teply (1949).

Bonner (1949*a*, 1949*b*), assuming that dilute dinitrophenol would have an inhibitory effect on phosphate transfers in living tissue, investigated its influence on the respiration and growth of *Avena* coleoptiles. He found that growth

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was inhibited while respiration was stimulated. He found also that adenylic acid increased both respiration rate and growth. From this he concluded that high-energy phosphate acceptors normally limit respiration in *Avena* coleoptiles and that dinitrophenol acts in respiration as an "effective substitute for adenylic acid and inorganic phosphate," but does not allow the transfer of energy-rich phosphate to growth processes. Newcomb (1950) has shown that dinitrophenol stimulates oxygen uptake in tobacco callus tissue.

In this paper, experiments designed to examine the effect of dinitrophenol on respiration and accumulation will be described. If the assumption that dinitrophenol can prevent transfer of phosphate groups from respiration in the cell is correct, then dinitrophenol can be used to determine whether phosphorylations are involved in the accumulation mechanism. It was realized that phosphorylations might be involved in two ways:

(a) directly or indirectly in the accumulation mechanism itself,

(b) indirectly in maintaining cell organization, in particular the resistance of the cytoplasm, which prevents the leakage of accumulated salt. The observed accumulation rate at any time must be the difference between a rate of uptake and a rate of leakage (Krogh 1946; Robertson and Wilkins 1948). Hence the experiments to be described deal not only with the effect of dinitrophenol on accumulation but also with the effect of dinitrophenol on leakage.

II. MATERIALS AND METHODS

Xylem parenchyma from carrot root, *Daucus carota* L. was cut into discs and washed for at least 96 hours by the methods described in earlier papers of this series (Robertson and Turner 1945). In some experiments discs were weighed the day before the experiment, placed in distilled water in small flasks, and aerated overnight. They were then blotted with filter paper and transferred to the experimental vessels. This procedure, by eliminating weighing on the day of the experiment, minimized the handling and shortened the period for settling down.

For one series of experiments, sets of 80 discs were taken immediately after cutting and threaded on fine silver wire, each disc being separated from its neighbour by a small glass bead. These were then washed and aerated in the usual manner.

(a) Respiration

The respiration was measured by standard Warburg technique. The vessels were shaken at 100 oscillations per minute in a thermostat at 25 or 27°C.

One gram of tissue and a total of 5 ml. solution was used in the vessels. The reagent to be tested was placed in the side-arm. When this was tipped into the vessel, the same volume of distilled water was tipped into the controls. When potassium cyanide was to be added, the vessels were removed from the manometers and the required amount of potassium cyanide added to the solution in contact with the tissue. The potassium hydroxide in the centre tube of the vessel was replaced with potassium hydroxide containing the appropriate quantity of potassium cyanide (Krebs 1935). When carbon monoxide

was used, the technique was the same as that described by Weeks and Robertson (1950); nitrogen-oxygen mixtures with the same oxygen concentration as the carbon monoxide-oxygen mixtures were used on replicate sets of tissue to determine the effect of low oxygen on the respiration rate.

Respiration results are expressed as cu. mm. O_2 /hr./g. fresh wt. Where dinitrophenol was added, the respiration rates were compared one and a half hours after the addition.

(b) Salt Uptake

Salt uptake was measured by following the changes either in conductivity or in chloride concentration, in the solution surrounding the tissue. In most experiments, the weighed discs were placed in the solution in 50 ml. florence flasks which were held in a thermostat on a carrier attached to the Warburg shaker. The tissue volume ratio was varied according to the requirements of the experiment; not less than 2 g. of tissue were used. In the series of experiments with discs threaded on silver wire, the threaded discs were placed in the solution in 100 ml. florence flasks in a thermostat and a continuous current of air bubbled through the solution. The air was passed through towers of water at the temperature of the thermostat before passing through the vessels to ensure that no water would be lost by evaporation.

In the conductivity experiments, a conductivity pipette was fitted in the cork of each flask and samples withdrawn periodically from the solution surrounding the discs, the conductivity measured, and the sample returned. Replication between sets of discs was good.

When changes in chloride concentration were to be determined, seven or more sets of discs were used for each treatment. The solution was poured off the first set after half an hour, off the second set after one hour, and so on. Samples of the solution were analysed for chloride by the indirect method with silver nitrate and thiocyanate.

Salt accumulation is expressed as g. mol. salt accumulated/hr./g. fresh wt. Where g. mol. salt could not be estimated (as in the very dilute solutions resulting from leakage), results are expressed in conductivity units, mhos/hr./g. fresh wt.

(c) Leakage of Phosphate

In some experiments, the external solution was analysed to determine how much phosphate leaked from the tissue in the presence of dinitrophenol. Phosphate was determined colorimetrically after the formation of phosphomolybdate.

III. RESULTS

(a) Respiration

The results of a typical respiration experiment are given in Figure 1; dinitrophenol in four concentrations was added to tissue in distilled water and in 0.05M potassium chloride. The stimulation of respiration by dinitrophenol

was marked in all but the set of discs in water to which the lowest concentration of dinitrophenol was added. The stimulation of the respiration occurred within 30 minutes of the addition of the dinitrophenol.

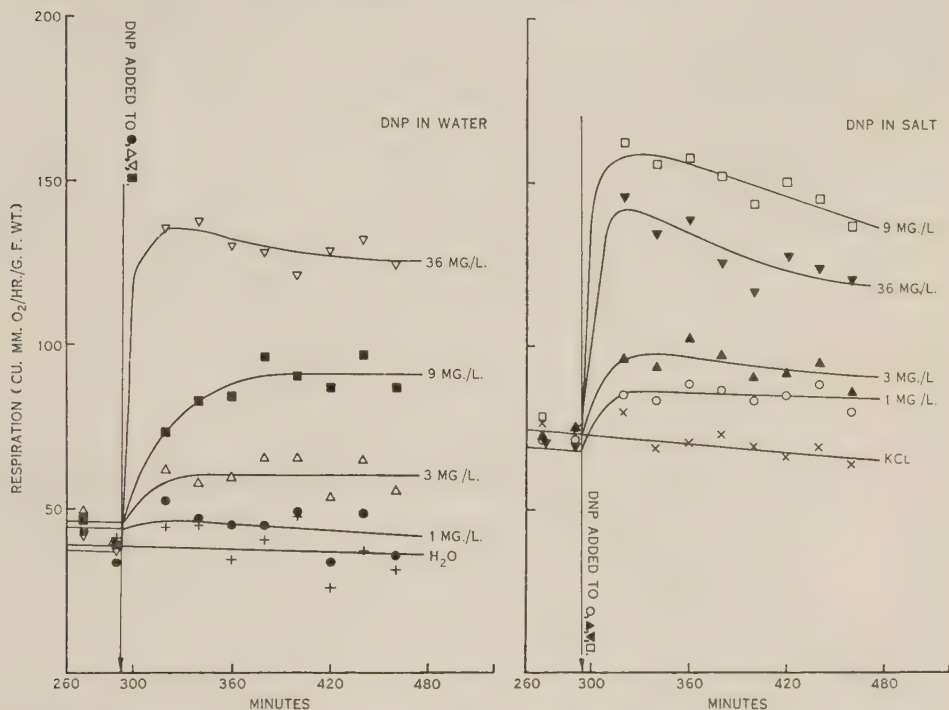


Fig. 1.—Effect of four different concentrations of dinitrophenol (DNP) on the respiration rate of carrot tissue in water and in 0.05M KCl solution. The respiration rates in water and in salt had been established before the dinitrophenol was added.

The effect of concentration of dinitrophenol in the experiment just described and in other experiments is summarized in Figure 3. Here the dinitrophenol respiration, i.e. the respiration rate in dinitrophenol over and above that in water or in salt $1\frac{1}{2}$ hours after the addition of dinitrophenol, is plotted against concentration of dinitrophenol. At low concentrations of dinitrophenol, the magnitude of the dinitrophenol respiration is much the same in water and in salt. In higher concentrations, whereas the dinitrophenol respiration in water is still increasing with concentration at 36 mg./l. dinitrophenol, it reaches its maximum in salt at about 9 mg./l. and then begins to decrease as the concentration increases. The stimulatory effect seems to be specific to 2,4-dinitrophenol, as *o*-nitrophenol, *p*-nitrophenol, and picric acid (2,4,6-trinitrophenol) did not affect respiration rate in the same concentrations.

(b) Accumulation

A typical experiment on the effect of dinitrophenol on accumulation is shown in Figure 2. When the dinitrophenol was added to the accumulation

vessels, a small amount of concentrated potassium chloride was added to offset the dilution effect*. The discs used in this experiment were from the same batch as those used in the respiration experiment, but had been cut for a longer period. The dinitrophenol inhibited the uptake of ions as judged by the conductivity change in the external solution, the effect of dinitrophenol in each concentration becoming apparent immediately after the addition.

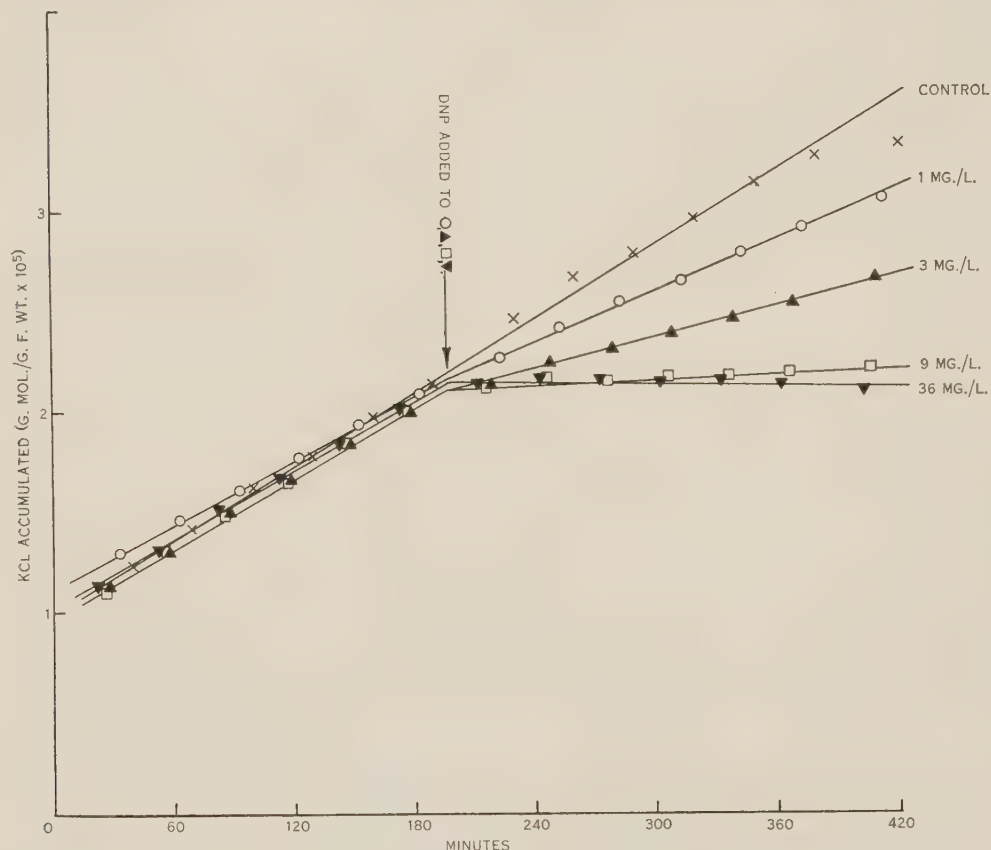


Fig. 2.—Effect of four different concentrations of dinitrophenol (DNP) on the amount of potassium chloride accumulated by carrot tissue in 0.05M KCl solution.

In all experiments, summarized in Figure 3, inhibition of accumulation increased sharply with increasing concentration of dinitrophenol; at concentrations above about 20 mg./l. there was a leakage of ions.

Since in these experiments the inhibition of accumulation had been determined from the conductivity change in the external solution, an experiment was carried out to determine whether the uptake of chloride itself was inhibited.

* This was not considered necessary in the Warburg vessels, since the concentration of chloride used would be adequate to ensure the full salt respiration (Robertson and Wilkins 1948).

The results of this experiment are given in Figure 4. Uptake was measured both by the change in conductivity and the change in chloride concentration. Fourteen sets of discs were taken; seven sets were placed in 0.05M potassium chloride and the remaining seven sets were placed in 0.05M potassium chloride with dinitrophenol (9 mg./l.). Samples for conductivity and chloride determinations were taken from each replicate at the required time. The conductivity results showed a complete inhibition of accumulation by dinitrophenol, after the initial uptake due to the physical equilibration. The chloride results indicated that, apart from the initial uptake, a very small amount of chloride entered over a short period, but this may not be significant. These results show that inhibition judged from conductivity determinations is an inhibition of the uptake of chloride itself, and is not due to an appreciable increase in leakage of other ions into the external solution.

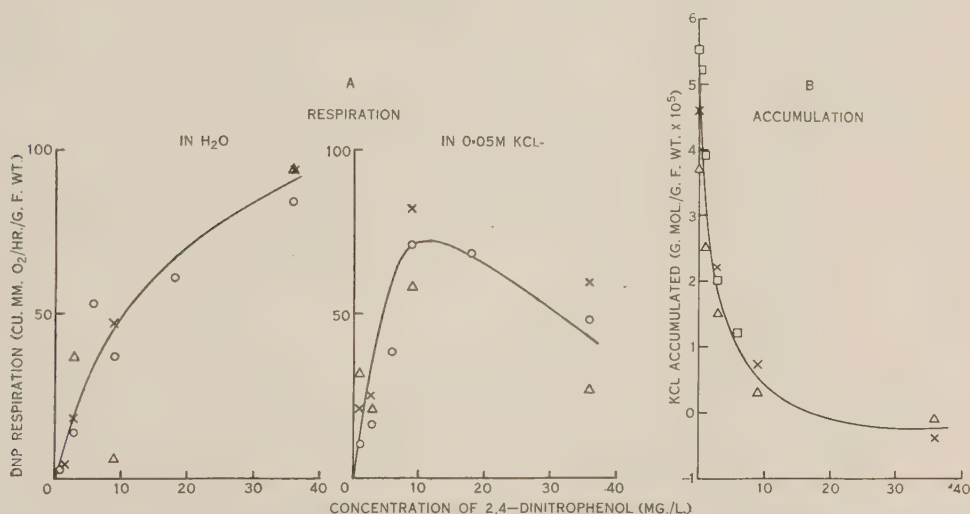


Fig. 3.—Relationship between concentration of dinitrophenol (DNP) and (A) the rate of the dinitrophenol respiration (i.e. the rate in DNP over and above that in water or in salt), and (B) amount of accumulation in carrot tissue; ×, O, and △, discs from one batch of carrots, respectively 144, 264, and 312 hr. from cutting; □ from another batch of carrots, 96 hr. from cutting.

(c) Leakage

In several experiments the change in conductivity produced by discs in water and in dinitrophenol was followed. This gave an estimate of the total loss of all electrolytes from the tissue. There was little or no leakage in water. Leakage in dinitrophenol was small but was always greater than that in water. If tissue that had been accumulating salt for some time was transferred to water or to dinitrophenol, the rate of leakage was greater than when the discs had had no pretreatment in salt. This can be seen from the results of the experiment given in Figure 5. Ten sets of discs were taken. Five sets were placed in water or dinitrophenol at four concentrations, 1, 3, 9, and 36 mg./l., and the conductivity changes followed. The remaining five sets

were placed in 0.05M potassium chloride and the conductivity changes followed for four hours. At the end of the four hours, the salt was drained off, the discs rinsed three times in distilled water, which was then drained off, and the required volume of water or dinitrophenol added. Other work has shown that this rinsing is sufficient to remove most of the salt from the intercellular spaces and cell surfaces. The rates of leakage with and without pretreatment in salt are plotted against concentration of dinitrophenol.

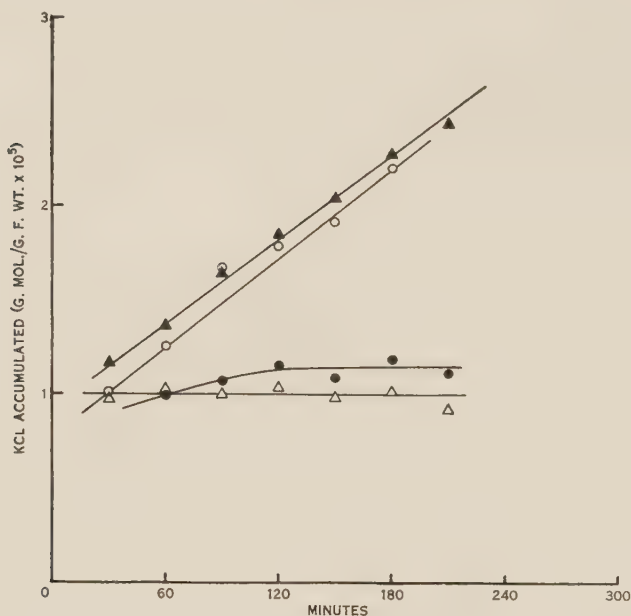


Fig. 4.—Inhibition of accumulation by DNP as measured by conductivity change and also by chloride change in the external solution (0.05M KCl). The upper curves show the amount of KCl accumulated from 0.05M KCl; ▲ from conductivity method; ○ from chloride method. The lower curves show the amount entering from 0.05M KCl in the presence of dinitrophenol (9 mg./l.); △ from conductivity method; ● from chloride method.

Though leakage in dinitrophenol was greater than in water, it did not account for the inhibition of accumulation observed. In several experiments the leakage in water and in dinitrophenol and the uptake in salt and in salt with dinitrophenol were determined in replicate sets of tissue. The results of one such experiment (discs threaded on wire) are given in Figure 6. The rate of leakage into water containing dinitrophenol was small compared with the difference between the rates of uptake from potassium chloride and from potassium chloride containing dinitrophenol. In some experiments, the leakage measured this way accounted for more of the difference between the normal and the inhibited accumulation, but greatest conductivity change in the external

solution (water containing dinitrophenol) due to loss of ions from the tissue accounted for only two-thirds of the difference and in most experiments for much less.

In four experiments, leakage from discs transferred to water from salt with dinitrophenol was not very different from that after transfer from salt alone, whether the dinitrophenol had caused partial inhibition of accumulation, complete inhibition of accumulation, or leakage. In the fifth experiment, the rate of leakage was greater after transfer from salt with dinitrophenol (36 mg./l.) than after transfer from salt alone. Even so, the rate of leakage into water accounted for only about 25 per cent. of the observed inhibition of accumulation in salt with dinitrophenol.

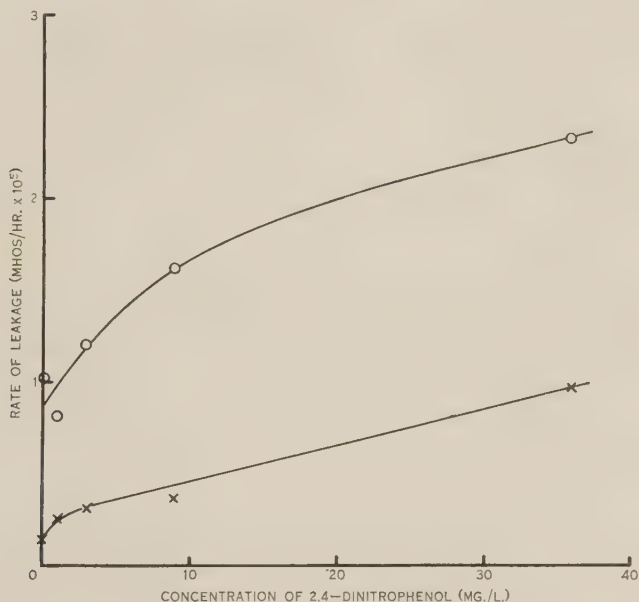


Fig. 5.—Rates of leakage from carrot tissue, measured by the increasing conductivity of the external solution, as a function of concentration of dinitrophenol; X, no pretreatment; O, pretreatment in 0.05M KCl.

The effects on respiration of these transfers from salt to water and to dinitrophenol were examined. Respiration rates of tissue in water, in dinitrophenol, and in salt were determined; some sets of discs were then transferred from salt to water and to dinitrophenol and the changes in respiration rate followed. Respiration rates in water and in dinitrophenol were similar to those in Figure 1. The usual salt respiration was observed. Transfer from salt to water resulted in a decrease in respiration rate though the rate was still higher than that of the control in water. The respiration rates of sets of discs transferred from salt to dinitrophenol rose (at 36 and 9 mg./l.) or fell (at 3 and 1 mg./l.) to approximately the level of the respiration rates of their replicate sets of discs, which had had no pretreatment in salt.

(d) Loss of Phosphate

Since it has been shown by Teply (1949) that dinitrophenol has a specific effect in liberating inorganic phosphate from the cyclophorase system, the loss of phosphate from the tissue in the presence of dinitrophenol was examined in one experiment. It was found that tissue in water or potassium chloride solution lost little phosphate to the external solution over a period of five hours.

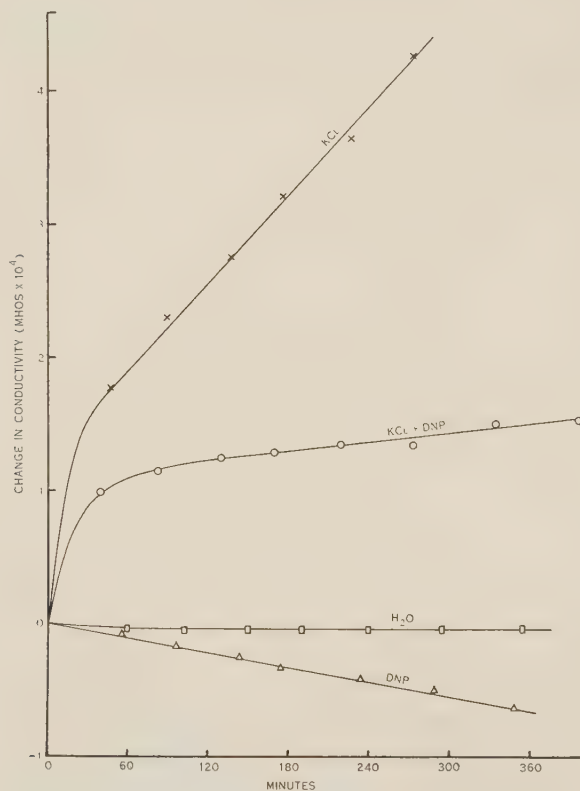


Fig. 6.—Uptake (positive) and loss (negative) of ions in carrot tissue in: 0.05M KCl, ×; 0.05M KCl with DNP (36 mg./l.), O; water, □; water with DNP (36 mg./l.), △. Results are expressed as conductivity change in the external solution.

In the presence of dinitrophenol, however, a measurable amount of phosphate was lost and this increased with time. The results are shown in Table 1. The conductivity change in the dinitrophenol solution was examined simultaneously; if the phosphate leaking were regarded as having the conductivity of sodium dihydrogen phosphate, it would account for only about 4.5 per cent. of the total conductivity change due to leakage in dinitrophenol. Hence although some phosphate ions were lost in the presence of dinitrophenol, they did not account for more than a small fraction of the total leakage.

(e) Comparison of the Effects of Dinitrophenol and Potassium Cyanide

The results of accumulation experiments in which dinitrophenol and potassium cyanide were added both separately and simultaneously are given in Table 2. It can be seen that:

(1) Dinitrophenol either partly inhibits accumulation or completely inhibits accumulation and some ions may leak from the tissue,

(2) Cyanide either partly inhibits accumulation or completely inhibits accumulation and ions do not leak from the tissue,

(3) Dinitrophenol and cyanide together inhibit accumulation and ions may leak slowly from the tissue.

TABLE 1

LOSS OF PHOSPHATE FROM TISSUES IN DIFFERENT SOLUTIONS COMPARED WITH CHANGES IN THE SPECIFIC CONDUCTIVITY OF THE EXTERNAL SOLUTIONS

Time (hr.)	External Solution							
	H ₂ O		DNP		KCl		KCl + DNP	
	Δ Sp. Cond. (mhos $\times 10^5$)	P (mg./ml. $\times 10^4$)	Δ Sp. Cond. (mhos $\times 10^5$)	P (mg./ml. $\times 10^4$)	Δ Sp. Cond. (mhos $\times 10^5$)	P (mg./ml. $\times 10^4$)	Δ Sp. Cond. (mhos $\times 10^5$)	P (mg./ml. $\times 10^4$)
0	0	0	0	0	0	0	0	0
1	3.2	1.2	2.9	3.0	-22.4	0.6	-14.4	3.6
2	4.2	1.0	5.6	4.4	-25.3	0.0	-14.8	6.2
4	6.2	1.0	10.1	7.4	-35.4	0.6	-12.6	11.4
5	8.0	1.0	12.5	—	-37.6	0.0	-13.0	16.2

When cyanide (0.001M) was added to tissue in dinitrophenol or potassium chloride with dinitrophenol, the respiration was rapidly reduced to the same level as that of tissue in water with cyanide and salt with cyanide. This level varied in tissue from different carrots. The respiration rate in water might be unaffected, partly inhibited, or slightly stimulated by cyanide.

Leakage of ions from tissue in water, cyanide (0.001M), and dinitrophenol (36 mg./l.) was compared in one experiment. The results are given in Figure 7. There was only a slight leakage in water and a slightly greater leakage in cyanide, which went on for more than five hours, after which the rate of leakage in cyanide began to increase; the rate had become quite rapid by the end of the experiment. The rate of leakage in dinitrophenol was about four times that in water. Cyanide was added to one of the two sets of tissue in dinitrophenol after four hours. The rate of leakage decreased considerably though it was still greater than the rate of leakage in water.

In one experiment, tissue was transferred from potassium chloride (0.05M) to water, cyanide (0.001M), dinitrophenol (36 mg./l.), and cyanide with dinitrophenol. After the transfer four readings were taken and then the vessels

were left shaking overnight and further readings taken in the morning. The results are shown in Figure 8. There was a slow leakage in water. In cyanide and cyanide with dinitrophenol there was at first a small uptake (initial uptake) and after this a slow leakage. Overnight, however, the leakage from tissue in cyanide ceased whereas the rate of leakage in cyanide with dinitrophenol increased. In dinitrophenol the rate of leakage was fairly high and continued at the same rate overnight.

TABLE 2
EFFECTS OF CYANIDE AND DINITROPHENOL ON THE ACCUMULATION RATE OF
POTASSIUM CHLORIDE IN CARROT TISSUE

Experi- ment	Time from Cutting (hr.)	Replicate Number	Accumulation Rate (g. mol./g./hr. $\times 10^5$)			
			KCl*	KCl* + DNP†	KCl* + KCN†	KCl* + KCN† + DNP†
NL1	144	1	0.36			
		2	0.38		0.25	
		3		0.21		
		4		0.21		0.03
		5	0.32	0.18		
		6	0.38	0.17		0.03
NL2	168	1	0.38			
		2	0.41		0.14	
		3	0.38	0.15		
		4	0.40	0.17		0.00
		5		0.16		
		6		0.20		0.03
BE1	96	1	0.38			
		2	0.52	—0.12		
		3	0.47			0.00
		4			0.07	—0.05
		5	0.65		0.04	
		6		—0.06		—0.02

* 0.05M.

† In NL1 and NL2, 3 mg./l; in BE1, 36 mg./l.

‡ 0.001M.

In the same experiment the rates of leakage in water, after pretreatment in salt (0.05M potassium chloride), salt with cyanide, salt with dinitrophenol, and salt with cyanide and dinitrophenol, were measured. Pretreatment in salt plus cyanide resulted in a slow leakage; the rate of leakage in water after pretreatment in salt with cyanide and dinitrophenol was greater, but not as great as that after pretreatment in salt with dinitrophenol alone.

(f) Effects of Dinitrophenol and Carbon Monoxide

The cyanide inhibition of the respiration stimulated by dinitrophenol suggests that this respiration, like the salt respiration, proceeds via the cytochrome-

cytochrome oxidase system. To establish whether the cytochrome oxidase system was involved, carbon monoxide, a specific inhibitor of this oxidase, was used in both light and darkness. A typical experiment is illustrated in Figure 9. A large increase in respiration rate was observed after the addition of the dinitrophenol (6 mg./l.). When the steady respiratory drift had been established, the air in some Warburg flasks was replaced by 93.5 per cent. CO-6.5 per cent. O₂, and respiration rates were then measured in both light and darkness. Carbon monoxide strongly inhibited the dinitrophenol respiration in

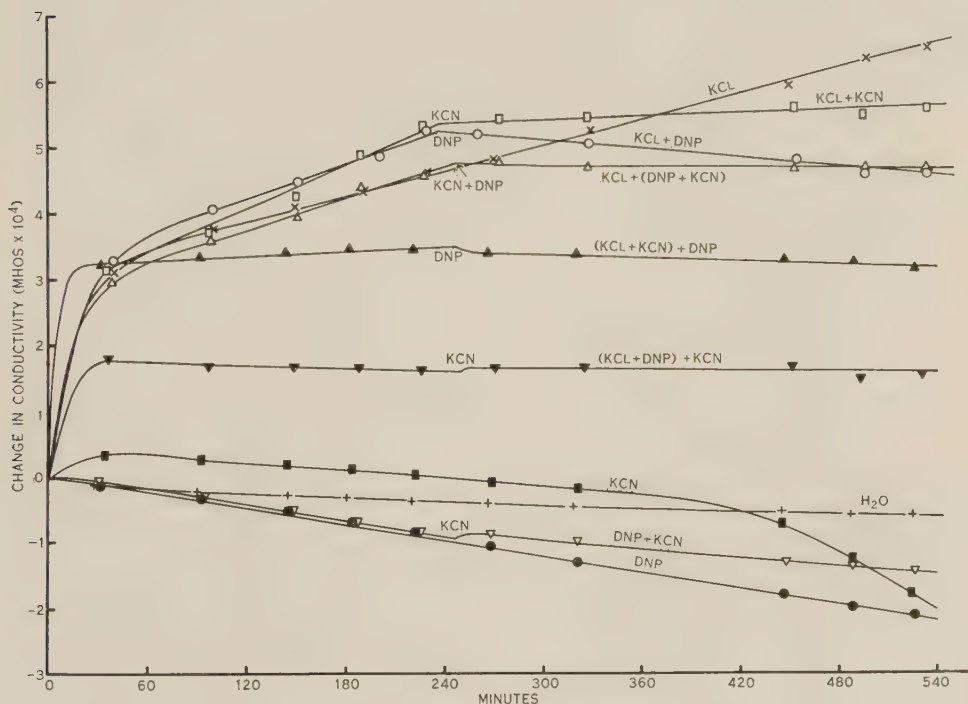


Fig. 7.—Effects of dinitrophenol (32 mg./l.) and of potassium cyanide (0.001M) on leakage in water and in potassium chloride solution (0.05M) with time. Results for uptake (positive) and leakage (negative) are expressed as change in conductivity of the external solution.

darkness, and bright light almost completely reversed this inhibition if allowance is made for the drift due to the low oxygen concentration. The effects of the gas mixtures on the respiration in water were also investigated but the results are not shown here; the respiration in water was depressed by CO in darkness to almost the same level as that in dinitrophenol, and was almost unaffected by low oxygen concentration. The small carbon monoxide inhibition was completely reversed by light. These results show conclusively that cytochrome oxidase is responsible for part of the respiration in these carrots, both in dinitrophenol solutions and in water, and that all the dinitrophenol respiration is mediated by cytochrome oxidase.

IV. DISCUSSION

From the above results it may be concluded that:

(1) Dinitrophenol inhibits salt accumulation and at high concentrations may even cause a leakage of ions from tissue in salt.

(2) Dinitrophenol increases the leakage of ions from tissue in water and from tissue transferred from salt to water; even if dinitrophenol caused the same amount of leakage from tissue in salt (though this leakage would tend to be less because the concentration gradient would be less), it would not account for the inhibition of accumulation as determined by the conductivity method.

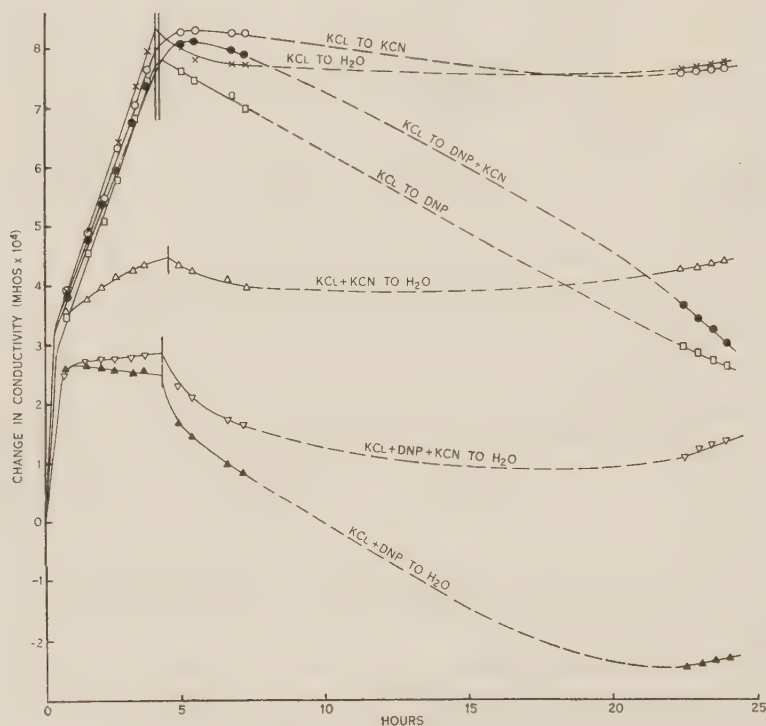


Fig. 8.—Effects of dinitrophenol (32 mg./l.) and potassium cyanide (0.001M) on leakage over long periods after pretreatment with 0.05M KCl, with KCl and KCN, with KCl and DNP, and with KCl, KCN and DNP. Results are expressed as change of conductivity of the external solution.

(3) Dinitrophenol stimulates the respiration of tissue in water and of tissue in salt; this stimulated respiration is sensitive to cyanide and is light-reversibly inhibited by carbon monoxide.

(4) Cyanide does not cause any appreciable leakage of ions from tissue in water at least for several hours, and apparently retards leakage from tissue in dinitrophenol.

(5) Cyanide inhibits salt accumulation and during the period of an experiment causes no leakage of ions from tissue in salt.

Thus dinitrophenol, like salt, allows more active functioning of the cytochrome system and its effect is additive to the salt effect; in spite of this, accumulation is inhibited. It has been shown both by the measurement of leakage rates and by the measurement of chloride uptake that the inhibition is not accounted for by a leakage of electrolytes that have previously been accumulated.

Hence it must be concluded that dinitrophenol either inhibits the transport mechanism itself or increases leakage only in the critical region across which the transport mechanism operates, so that ions being moved into the cell leak back at such a rate that there is no net accumulation.

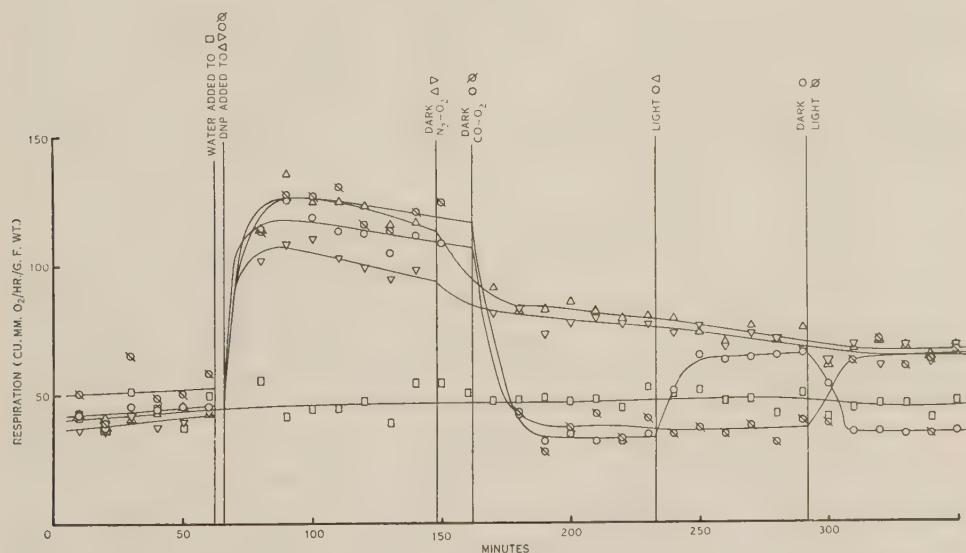


Fig. 9.—Effect of carbon monoxide on the respiration of carrot tissue in KCl solution (0.05M) with and without dinitrophenol (6 mg./l.); gases contained 6.5 per cent. O_2 .

(a) Effect on Accumulation

Whatever the mechanism of the action of dinitrophenol, there is ample evidence (Loomis and Lipmann 1948; Cross *et al.* 1949; Teply 1949) that it interferes with the transfer of energy-rich phosphate from respiratory reactions to other processes. There is as yet insufficient evidence to decide how an inhibition of energy-rich phosphate transfers might interfere with the ion transport mechanism.

It may be that, in the presence of dinitrophenol, the cytochrome system, though operating at a greater rate in the presence of dinitrophenol, is no longer in a position to transport anions. There is increasing evidence, particularly that of Green, Loomis, and Auerbach (1948) and of Hogeboom,

Schneider, and Pallade (1948), that the succinoxidase system is associated with mitochondria. Similar conclusions about plant succinoxidase have been reached by duBuy, Woods, and Lackey (1950). Further, interference with the normal energy-rich phosphate transfer system brings about some loss of function and integrity in the mitochondrial particle (Teply 1949; Harman 1950*a*). Hence it may be that the accumulation occurs only if the cytochrome oxidase system is organized in the intact mitochondrion and that the dinitrophenol interferes with this organization. Though Harman (1950*b*) did not find any accumulation of sodium or potassium in the mitochondria, Mullins (1940) found that both phosphate and potassium accumulated in the protoplasmic granules of *Nitella*.

Alternately, the transport by the cytochrome system may be only one stage in a mechanism consisting of several stages in series, at least one of which requires a phosphorylation. In this hypothesis some of the energy for the transport of ions would be derived from unstable phosphate esters, as suggested by Davies and Ogston (1950) in explaining the secretion of hydrochloric acid by the gastric mucosa. Finally it is possible that the cytochrome system plays no direct part in the accumulation mechanism; if this were so a new explanation of the quantitative relation between salt accumulation and salt respiration (Robertson and Wilkins 1948) would have to be sought.

(b) Effect on Respiration

It is not yet clear how dinitrophenol increases the respiration rate, though the suggestion that it liberates free phosphate which has been limiting the rate of respiration is plausible (Teply 1949). It seems likely that it would have an effect in carrot tissue similar to that in *Avena* coleoptile (Bonner 1949*b*); both tissues show respiration stimulated by adenylic acid.

The respiration of carrot tissue, both in water and in salt, was stimulated by even the highest concentration of dinitrophenol used (36 mg./l.), while Bonner (1949*b*) found that the respiration of *Avena* coleoptiles, though stimulated at low concentrations (below 6-7 mg./l.), was almost completely suppressed at higher concentrations (10 mg./l.). While this difference may be inherent in the tissue, it may be only apparent, since Bonner was using solutions buffered at pH 4.5. In most of our experiments no buffer solution was added but the pH of the external solution changed only from pH 5.0 to 5.5. An experiment with carrot tissue in dinitrophenol (9 mg./l.) buffered at pH 3.5, 5.5, and 7.5 with citrate-phosphate showed that the effect of dinitrophenol was influenced by the pH level. At pH 3.5 respiration was almost completely suppressed, whereas at pH 5.5 and 7.5 the respiration was stimulated. At low pH values, owing to the suppression of ionization of both the dinitrophenol and the weak electrolytes of the cytoplasm, the concentration of dinitrophenol in the cell would probably be greater than at higher pH values.

Bonner found that the inhibitory effect of dinitrophenol on respiration was not shown if pyruvate was supplied as a respiratory substrate. He suggested that, at high concentrations, dinitrophenol interferes with the normal

production of pyruvate from hexose, a process known to require adenosine-triphosphate, formation of which may be inhibited by dinitrophenol. Some similar effect of dinitrophenol may account for the submaximal effect of high concentrations (above about 10 mg./l.) on the respiration of tissue in salt. The effect of inhibition of the production of pyruvate would be expected to be more marked in tissue in salt than in tissue in water, since presumably the rate of utilization of pyruvate is greater in salt than in water.

(c) *Effect on Leakage*

Leakage of ions from carrot tissue in water is very small. When dinitrophenol is applied to the tissue, the leakage is increased. The reason for this increase is not yet known. If the resistance in the cell to ion diffusion were dependent for maintenance wholly on phosphorylations, then a greater increase in leakage in dinitrophenol would be expected. The fact that the resistance does not decrease markedly in the presence of dinitrophenol may mean that a continuous supply of energy-rich phosphate is not necessary for its maintenance or that sufficient energy-rich phosphates are still available.

The ions appearing in the external solution have not yet been fully identified and may be ions liberated from the cytoplasm owing to some effect of the dinitrophenol. Phosphate, which may correspond to the "gel phosphate" liberated by dinitrophenol from the cyclophorase system (Green *et al.* 1949; Teply 1949), is apparently liberated, but accounts for only a small fraction of the total ions lost to the external solution.

Another possible explanation of the leakage is that the dinitrophenol, without having much effect on the overall cytoplasmic resistance, inhibits the mechanism that normally operates to transport back to the cell interior the small quantity of ions that escape through the high-resistance region.

Dinitrophenol alone increases leakage more than dinitrophenol with cyanide. Leakage is also greater in water after transfer from salt with dinitrophenol than after transfer from salt with dinitrophenol and cyanide. This may be a real difference due to some antagonism between the dinitrophenol and the cyanide and might, for instance, be connected with the effects of these substances on the mitochondria. The problem of leakage obviously requires more thorough investigation.

It must be concluded that the dinitrophenol inhibits some process intimately connected with accumulation; it does not inhibit the cytochrome-cytochrome oxidase system; hypotheses of salt accumulation must allow for this conclusion.

V. ACKNOWLEDGMENTS

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VI. REFERENCES

- BONNER, J. (1949a).—*Amer. J. Bot.* **36**: 323.
BONNER, J. (1949b).—*Amer. J. Bot.* **36**: 429.
CROSS, R. J., TAGGART, J. V., COVO, C. A., and GREEN, D. E. (1949).—*J. Biol. Chem.* **177**: 655.
DAVIES, R. E., and OGSTON, A. G. (1950).—*Biochem. J.* **46**: 324.
DUBUY, H. G., WOODS, M. V., and LACKEY, M. D. (1950).—*Science* **111**: 572.
GREEN, D. E., ATCHLEY, W. A., NORDMANN, J., and TEPLY, L. J. (1949).—*Arch. Biochem.* **24**: 359.
GREEN, D. E., LOOMIS, W. F., and AUERBACH, V. H. (1948).—*J. Biol. Chem.* **172**: 389.
HARMAN, J. W. (1950a).—*Exp. Cell Res.* **1**: 382.
HARMAN, J. W. (1950b).—*Exp. Cell Res.* **1**: 394.
HOAGLAND, D. R. (1944).—"Lectures on the Inorganic Nutrition of Plants." (New York.)
HOGEBOOM, G. H., SCHNEIDER, W. C., and PALLADE, G. E. (1948).—*J. Biol. Chem.* **172**: 619.
KREBS, H. A. (1935).—*Biochem. J.* **29**: 1620.
KROGH, A. (1946).—*Proc. Roy. Soc. B* **133**: 140.
LOOMIS, W. F., and LIPMANN, F. (1948).—*J. Biol. Chem.* **173**: 807.
LUNDEGARDH, H. (1945).—*Ark. Bot.* **32A** (12): 1.
MULLINS, L. J. (1940).—*Proc. Soc. Exp. Biol. N.Y.* **45**: 856.
NANCE, J. F. (1949).—*Science* **109**: 174.
NEWCOMB, E. H. (1950).—*Amer. J. Bot.* **37**: 264.
ROBERTSON, R. N., and TURNER, J. S. (1945).—*Aust. J. Exp. Biol. Med. Sci.* **23**: 63.
ROBERTSON, R. N., and WILKINS, MARJORIE J. (1948).—*Aust. J. Sci. Res. B* **1**: 17.
TEPLY, L. J. (1949).—*Arch. Biochem.* **24**: 383.
WEEKS, D. C., and ROBERTSON, R. N. (1950).—*Aust. J. Sci. Res. B* **3**: 487.

MEMBRANE POTENTIAL DIFFERENCES IN BEAN ROOTS

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Summary

Various theories for the formation of electric potential differences in plant organs are outlined and the present investigation placed in relation to them.

Broad bean root tips behave as hydrogen electrodes in dilute solutions of the mineral acids HCl, HNO₃, and H₂SO₄ in a limited pH range, as shown by the change in p.d. between the root surface and the solution with change in H⁺ ion concentration.

When the root tips are placed in neutral salt solutions of concentration between 0.1 and 0.0001N, there is a linear change in potential with logarithmic change in salt concentration. The solution is more positive for smaller concentrations. This is interpreted as a diffusion potential phenomenon, with greater mobility of the cation than the anion through the protoplasmic membrane. The ratios of mobility of cation and anion for four salts are calculated on this assumption.

When bean root tips are placed in buffer solutions with pH's in the range 3-5 and with a constant molar concentration of 0.1, there is no significant change in potential with pH.

There is a significant and nearly normal change in p.d. between root and solution for a change in KCl concentration from 0.01N to 0.1N when the KCl is at pH 3 or 5. Both buffered KCl and KCl + HCl bathing solutions were used.

The hypothesis is advanced that the p.d. between root and solution is the result of the diffusion of inorganic ions through or into the root protoplasmic membrane. The results are discussed in relation to Lundegardh's theory concerning the adsorption and exchange of cations at root surfaces.

I. INTRODUCTION

There has been a great deal of controversy in the last 10-20 years as to the origin of the electrical potential differences measured in biological systems. Several distinct theories have emerged which *seem* to be mutually exclusive, and in some cases apparently conflicting experimental results have appeared.

Lund and his co-workers (Lund and Kenyon 1927; Marsh 1928; Lund *et al.* 1947) having done extensive work on the electrical polarity of onion roots, *Avena* coleoptiles, Douglas fir, etc., postulate from the combined evidence that the "polarity potentials," or the main part of them, are oxidation-reduction potentials connected directly with the respiratory mechanism of the plant.

The redox theory has been criticized (Blinks 1949; Lundegardh 1940; Thomas 1939) partly on the grounds of experimental evidence and partly for

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theoretical reasons, it being thought that since noble metal electrodes were not used to measure the p.d. and are not present in the biological materials, a redox mechanism involving sources and sinks of electrons could not be directly responsible for the measured p.d.'s. However, this objection is not serious since it can be *assumed*, not unreasonably (Höber *et al.* 1947), that the plant contains loci that can act as oxidation-reduction electrodes. On the other hand, certain experiments of Thomas (1939) do not seem to conform to the redox hypothesis.

Blinks (1949), Osterhout (1936), and Hill and Osterhout (1937) find that membrane potentials appearing in large single-celled organisms can be explained as diffusion potentials of inorganic salts. The ratio of the mobilities of the cation and anion appear to be modified by the external protoplasmic membrane from the value for an aqueous solution.

Lundegardh (1940) postulates an alternative mechanism for the appearance of the membrane potentials in cereal roots. He has investigated the change in p.d. between root and surrounding solution with change in concentration of acid or salt in the solution and obtains results that he interprets as being due to an adsorption-exchange of cations on the root surface. Williams and Coleman (1950) also report cation exchange processes taking place on root surfaces. However, Lundegardh's results also seem consistent with the assumption of diffusion potentials, except for one particular experiment on the influence of the acidity of the bathing solution on the change of p.d. with concentration.

In view of the unsatisfactory position as regards the theories of bioelectric potentials (redox as opposed to diffusion, diffusion as opposed to adsorption-exchange potentials), it was thought advisable to make a start on the problem of the mechanism for the production of bioelectric potentials by repeating and extending some of Lundegardh's experiments to try to decide between the "diffusion" and "adsorption-exchange" theories. Such a research was also desirable in view of the apparent importance of salt concentration and pH in determining the measured p.d.'s in certain other experiments in this laboratory on plant organization.

The results given below in the main agree with those of Lundegardh but a disagreement has been found in the experiments on the change of p.d. with salt concentration at varied pH's. In consequence of this, the results when taken as a whole seem to be consistent with the assumption of diffusion potentials set up at the boundary between an acid-dissociated membrane and a surrounding solution.

However, it is emphasized that knowledge of the nature of the membrane potentials at any one region of a root surface does not permit conclusions to be drawn about the longitudinal p.d.'s that appear when equimolar contacting solutions are applied at two different locations on the root (Lund *et al.* 1947; Thomas 1939). Further experiments are in progress to elucidate the nature

of these 'p.d.'s. They are a function of at least two membrane p.d.'s which may themselves be different functions of salt concentration and pH, depending on the location of the two contacts.

Also, the radial p.d.'s between surface and inside of roots and hypocotyls (McAulay, Ford, and Hope, unpublished data) are no doubt composed of several phase boundary potential differences, one of which is that between protoplasmic membrane and contacting solution and is dealt with here. Other p.d.'s probably exist between protoplasm and cell sap and between different cells.

It is possible that all three mechanisms mentioned are concerned in bio-electric potentials, the measured p.d. being a function of one or more of them in single-celled organisms, others in non-liquid absorbing organs, and so on.

II. APPARATUS AND MATERIALS

Potential differences were measured with a valve millivoltmeter of high input resistance, the grid current being less than 10^{-12} A. The accuracy was about ± 2 mV. in the range 0-200 mV. The concentration of hydrogen ion in the salt, acid, and buffer solutions was measured with a valve pH meter, accurate to within at least 0.1 pH units.

The electrodes used were small 2N calomel half-cells with a 2N KCl/agar jelly bridge waxed into 6 mm. diameter glass contact tubes filled with 1N KCl/agar. The other ends of these were drawn out to approximately 100 μ diameter. These were moved in the region of the plant roots with screw micro-manipulators. The contact tip + electrode zero error when both tips were placed in any of the solutions used was always less than 1 mV.

The roots were held in a vertical position in a perspex box saturated with water vapour and with a flow of fresh air bubbling through a water reservoir at the base. The roots were illuminated with a spotlight and observed with a low power dissecting microscope.

Commercial broad bean (*Vicia faba* L.) roots were used as the experimental material in the experiments to be described. They were germinated by soaking in tap water 24 hours and planting in sphagnum in a glass-house at 20°C. Approximately two days after planting the roots were from 20 to 40 mm. long and were used at this stage. The diameter of the root about 10 mm. from the tip was between 1.5 and 2.5 mm.

The plants were handled by the seed coat in the transfer from sphagnum trough to saturated box and there allowed to stabilize for some minutes. The tip of the root was dipped into a polystyrene cup holding 3 ml. of solution, the cup being filled with an eye-dropper and emptied via a rubber tube connected to its base. During the whole series of experiments the temperature inside the box varied between 18.5°C. and 23.5°C.

III. RESULTS

(a) Response of the Potential of the Root Tip to Changing Concentration of Mineral Acids

It is shown that the root tip of the broad bean behaves as a hydrogen electrode in solutions of certain mineral acids of concentration less than about $10^{-3}N$. The potential of the root tip relative to the acid solution changes by approximately 60 mV. at $21^{\circ}C$. for a ten times change in acid concentration. As Lundegardh has pointed out, the high speed of response of the p.d. on the application of a new concentration points to the boundary between root tip and solution as the site of the changing p.d. Many experimenters assume the site to be the outer protoplasmic membrane of the epidermal cells.

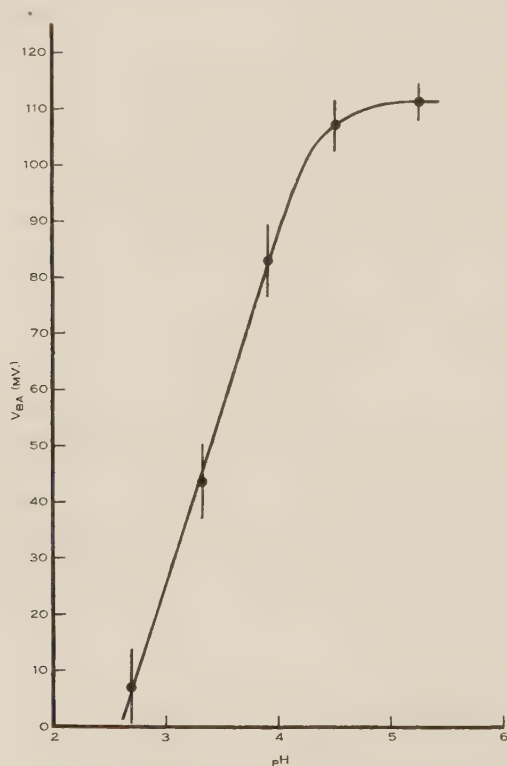


Fig. 1.—Variation of p.d. between KCl/agar contact (at *b*, 10 mm. from the root tip) and solution (*a*) with pH of HCl solution. The sign of the p.d. is that of *a* relative to *b*. The points plotted are the means and standard deviations for five experiments.

The graph of change in p.d. with change in pH for HCl concentrations in the range $pH = 2.5-5.5$ is shown in Figure 1. The points plotted are the averages and standard deviations for experiments on five roots. The acids

were applied to the root in increasing concentration. In the pH range 2.5-4.0 the equation to the graph is

$$V_{ba} = -155 - 60 \log_{10} [\text{HCl}] \text{ (mV.)} \dots \dots (1)$$

the sign being that of the solution *a* relative to *b*, a reference point. The curving over of the graph after pH 4 could be caused by evolved CO₂ from the root lowering the pH of the applied acid from the measured value. Equation (1) may be compared with the familiar Nernst equation for electrode potentials:

$$E = E_o - 58 \log_{10} [M^+] \text{ (mV. at } 20^\circ\text{C.)} \dots \dots (2)$$

Contact with a root was made about 10 mm. from the tip at "b" through 0.1N KCl/agar jelly and 1-2 mm. of the root tip was dipped into the cup containing the HCl solutions. The circuit was completed by dipping the second contact tip into the solution beside the root (contact "a"). Readings were taken at the beginning and end of one-minute intervals, the solution then being changed. Similar experiments with HNO₃ and H₂SO₄ gave results that are not statistically different from Figure 1.

The x-axis intercept may be thought to give the average pH of the outer layers of the protoplasm of the root tips, but little significance can be attached to the value (2.6) because of an unknown membrane potential between root and KCl/agar at *b*. There is, however, evidence that this unknown p.d. is small.

The maximum concentration of the acids applied was limited to a little greater than 10⁻³N because it was found that acid of about that concentration killed the roots on prolonged immersion. When strong acid is applied and the concentration decreased, graphs are obtained with an average slope of about 40 mV. per pH unit. This is possibly due to permanent damage caused by the initial application of acid of pH 2.7.

(b) Response of the Potential of the Root Tip to Changing Concentration of Neutral Salts

The salts KCl, NaCl, KNO₃, and NaNO₃ give an approximately linear relation between change in p.d. and log (salt concentration) when applied to the root tip in concentrations between 0.1N and 0.0001N. The graph of an average of 10 experiments with KCl is shown in Figure 2. The straight line has the equation

$$V_{ba} = -38 - 32 \log_{10} [\text{KCl}] \text{ (mV.)} \dots \dots (3)$$

which, if a diffusion type potential is assumed to be present, may be compared with

$$E = E_o - 58 \frac{u-v}{u+v} \log_{10} [\text{KCl}] \text{ (mV. at } 20^\circ\text{C.)} \dots \dots (4)$$

where *E* is the measured p.d., *u*, *v* the mobilities of cation and anion respectively in the protoplasmic membrane, and *E*_o a constant including the p.d. between the KCl/agar and the root surface at *b* and also a term containing the concentration of salts in the membrane. By identifying equations (3)

and (4), u/v , the ratio of the mobilities of cation and anion in the protoplasmic membrane, may be evaluated.

TABLE 1
RATIOS OF IONIC MOBILITIES

Salt	Average Slope of Graphs of p.d. vs. log [MA]	u/v Calc.	u/v in Aqueous Solutions at ∞ Dilution
KCl	32 mV./log unit	3.5	0.98
NaCl	28 mV./log unit	2.9	1.04
KNO ₃	25 mV./log unit	2.5	0.66
NaNO ₃	31 mV./log unit	3.3	0.70

Table 1 shows the average slopes of graphs for 10 experiments with each of KCl, NaCl, KNO₃, and NaNO₃ solutions, the calculated values of u/v , and for comparison, the ratios of mobilities in aqueous solutions at infinite dilution.

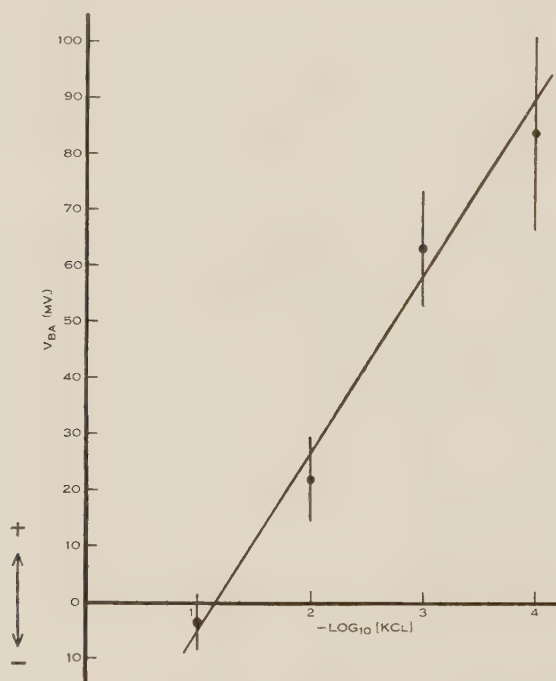


Fig. 2.—Variation of p.d. between root and solution with concentration of KCl, showing the means and standard deviations for 10 experiments. The sign of V_{ba} is that of a relative to b .

It is seen that, if the graphs are interpreted in this way, K^+ and Na^+ have a similar mobility in the membrane compared with Cl^- . In these experiments, there was no statistical difference in the results obtained when solutions were applied in increasing and in decreasing concentration.

(c) *Response of the Potential of the Root Tip to Changing pH in Buffer Solutions*

Experiments of the type described in Sections III (c) and III (d) were made to find if exchange of cations for H^+ ions (adsorbed to the root surface) takes place when the root is immersed in a salt solution. This process is the basis of Lundegardh's theory of salt adsorption (see Lundegardh 1940 and Section IV). It is obvious that such exchange would be regulated by the pH of the surrounding salt solution, since, for example, if the pH of root surface and solution were the same, H^+ ions would have little tendency to leave the root. Also, the potential difference between root and solution would depend upon the nature of the adsorbed cation (of the strength of the dipoles it forms), and this provides a means of testing the hypothesis.

TABLE 2
CHANGE IN P.D. WITH pH IN BUFFER SOLUTIONS

pH Change	4.9-4.5	4.5-3.9	3.9-3.5	3.5-3.1	3.1-2.5	2.5-1.9	1.9-1.5	1.5-1.1
P.d. change	+ 4	+ 4	0	- 2	- 6	- 2	0	0

When buffer solutions* in the pH range 3-5 and with equimolar salt concentrations are applied to the root tip in either increasing or decreasing pH, there is no characteristic change of p.d. with change in pH. There is, however, a general drift of the p.d. with time when the root is in any particular solution and this is enlarged upon in Section III (e). Table 2 records for a typical experiment the change in p.d. between root and solution for the

TABLE 3
CHANGE OF P.D. FOR A CONCENTRATION CHANGE FROM 0.01N TO 0.1N AT DIFFERENT pH'S

(i) Buffered KCl			(ii) KCl and HCl		
Root No.	δV at pH 3 (mV.)	δV at pH 5 (mV.)	Root No.	δV at pH 3 (mV.)	δV at pH 5 (mV.)
1	22	12	1	25	28
2	24	45	2	23	26
3	21	32	3	23	27
4	28	28	4	23	27
5	37	22	5	22	35
Mean	26.4	27.8	Mean	23.2	28.6
S.D.	± 5.8	± 10.8	S.D.	± 1.0	± 3.2

corresponding change in pH. It is seen that the p.d. changes are small and random and that the membrane potential is thus a function of total molar concentration (and time) rather than of hydrogen ion concentration.

* McIlvaine's buffer (citric acid plus disodium hydrogen phosphate) was made up to pH's in the range 3 to 5, the total molar concentration being about 0.1 for each solution.

(d) *Response of the Potential of the Root Tip to Change of KCl Concentration at Different pH's*

It is shown that the change in potential between root tip and solution for a ten-times change in concentration of KCl at pH's of 3 and 5 is very nearly the same. Table 3 shows the results of 10 experiments, five with buffered KCl and a second five with KCl acidified with HCl. The table records the change in V_{ba} for a change in KCl concentration from 0.01N to 0.1N, firstly at pH 3 and secondly at pH 5. In (i) the response is independent of pH while in (ii) the difference may be significant but δV at pH 3 is still large. It is seen that in these experiments the change of p.d. with change in salt concentration is not greatly influenced by the pH of the salt solution.

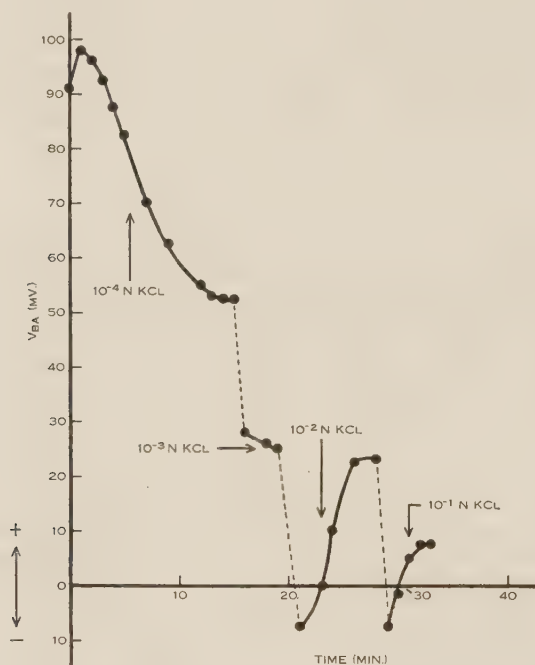


Fig. 3.—Variation of p.d. V_{ba} with time for different concentrations of KCl solution. The sign of the potential is that of a relative to b .

(e) *Change of the Potential of the Root Tip with Time for Various Concentrations of KCl*

Figure 3 is a typical graph of the time rate of change of V_{ba} for changing KCl solutions of concentration 0.0001, 0.001, 0.01, and 0.1N. The usual changes in p.d. are obtained on changing the concentration of the solution, but while the p.d. with the root tip in 10^{-3} N KCl is practically constant with time, the solution tends to become more negative when the root tip is in 10^{-4} N KCl and more positive when in 10^{-2} N, 10^{-1} N KCl.

From equation (4), if this expression is accepted as describing the p.d.: concentration relation, it is seen that if E is to vary for constant $[KCl]$ then either E_o or u/v must vary with time. Since the changes in p.d. obtained on changing the KCl concentration tenfold are comparable with those of Figure 2, u/v must be assumed constant. On the other hand, E_o contains terms including both the phase boundary p.d. at b and the salt concentration of the root phase. As the $10^{-1}N$ KCl/agar contact at b was established only some 5-10 min. before the time "zero" of Figure 3, the p.d. at b cannot be assumed constant and it is felt that a detailed interpretation of Figure 3 must be left until further experiments are made. Without consideration of the p.d. at b the p.d. variations in Figure 3 point to diffusion of salt into the root at outside concentrations of 10^{-1} and $10^{-2}N$ and back diffusion into the bathing solution at $10^{-4}N$.

This effect would have to be reconciled with existing theories for the accumulation of salt by root tissue from dilute solutions.

IV. DISCUSSION

The experiments described were made following and extending Lundegardh's (1940) to elucidate the nature of the phase boundary potential differences found between roots and solutions surrounding them. The results on the whole are consistent with the hypothesis of diffusion potentials set up at the interface between the solution and the root outer protoplasmic membrane.

The signs of the potential differences are such as to suggest that the cation has a greater mobility than the anion, with the salts investigated. The results indicate that the protoplasm is acid-dissociated with a pH in the region of 3. The data of certain of the experiments are in agreement with Lundegardh's but the crucial experiments of Sections III (c) and III (d) do not seem to conform to the adsorption mechanism and this is discussed in the following.

According to Lundegardh the potential of a root surface relative to a solution bathing it is derived from a difference in hydrogen ion concentration between the root and solution. If there has been a primary adsorption of hydrogen ions (from acid or pure water) to postulated free negative valencies in a monolayer, then the root will behave as a hydrogen electrode in solutions of pure acids. Also H^+ ions are said to be exchanged for metallic cations when the root is placed in a salt solution. The pH of the root surface, and thus its potential relative to the solution changes with varying concentration of salt.

The results given in Sections III (a) and III (b) are in accordance with this view but also with the hypothesis of a diffusion process in an acid-dissociated membrane. A concentration of free hydrogen ion of about $0.001N$ in the root phase accounts for the relation between p.d. and pH given in Figure 1 while selective permeability makes reasonable the relation between p.d. and salt concentration when the root tip is placed in a salt solution (Fig. 2 and Table 1).

Further, on the adsorption-exchange theory, the tendency of exchange of H^+ for M^+ in a salt solution should depend to a large degree on the pH of this

solution. Lundegardh reports that at low pH's (3-4) little exchange of H^+ for M^+ occurs, as reflected by the absence of change of p.d. for change in salt concentration at this acidity. However, in the experiments reported (Sections III (c) and III (d)) there was no significant change in p.d. for change in pH at a constant concentration of about 0.1M nor was there much difference in magnitude in the response to a change in KCl concentration from 0.01N to 0.1N at pH 3 and pH 5. This is at variance with Lundegardh's results and theory, and speaks once more for a diffusion type potential difference being present.

Similarly, the evidence given in Section III (e) showing the variation of the root p.d. with time in various concentrations of salt is consistent with a tendency towards the establishment of equilibrium between the ions in bathing solution and in the protoplasm by the mechanism of diffusion.

V. ACKNOWLEDGMENTS

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VI. REFERENCES

- BLINKS, L. R. (1949).—The source of the bioelectric potentials in large plant cells. *Proc. Nat. Acad. Sci. Wash.* **35**: 566-75.
- HILL, S. E., and OSTERHOUT, W. J. V. (1937).—Calculations of bioelectric potentials. II. The concentration potential of KCl in *Nitella*. *J. Gen. Physiol.* **21**: 541-56.
- HÖBER, R., *et al.* (1947).—"Physical Chemistry of Cells and Tissues." pp. 676. (P. Blakiston's Son & Co. Inc.: Philadelphia.)
- LUND, E. J., and KENYON, W. A. (1927).—Relation between continuous bioelectric currents and cell respiration. I. Electrical correlation potentials in growing root tips. *J. Exp. Zool.* **48**: 333-57.
- LUND, E. J., *et al.* (1947).—Bioelectric fields and growth. Ed. Austin. pp. 391. (University of Texas Press.)
- LUNDEGARDH, H. (1940).—Investigations as to the absorption and accumulation of inorganic ions. *Ann. Agric. Coll. Sweden* **8**: 233-404.
- MARSH, G. (1928).—Relation between continuous bioelectric currents, and cell respiration. IV. The origin of the electric polarity in the onion root. *J. Exp. Zool.* **51**: 309-25.
- OSTERHOUT, W. J. V. (1936).—Electrical phenomena in large plant cells. *Physiol. Rev.* **16**: 216-37.
- THOMAS, J. B. (1939).—Electric control of polarity in plants. *Rev. Trav. Bot. Néerland* **31**: 374-436.
- WILLIAMS, D. E., and COLEMAN, N. T. (1950).—Cation exchange properties of plant root surfaces. *Plant and Soil* **2** (3): 243-53.

FACTORS INFLUENCING THE FUNGISTATIC ACTION OF 8-HYDROXYQUINOLINE (OXINE) AND ITS METAL COMPLEXES

By BEVERLEY I. ANDERSON* and R. J. SWABY*

[Manuscript received January 2, 1951]

Summary

The fungistatic effect of oxine against *Aspergillus niger* is greatly reduced if the medium is first freed of cupric Cu, and toxicity increases with increasing amounts of Cu. The toxicity is overcome by lowering the pH of the medium even in the presence of quite large amounts of Cu.

Removal of ferrous Fe slightly reduces the fungistatic activity of oxine but deficiencies of Zn, Mn, or Mo have no effect. Oxine inhibition is not reversed by the addition of high concentrations of Co, Zn, Mn, Fe, or Cu and only partially by Mo.

The toxicity of seven chelators was tested in the presence and absence of Cu and, of these, four showed an increase in fungistatic action in the presence of Cu.

These findings are discussed in relation to the possible mode of action of oxine and its metal complexes on fungal cells.

I. INTRODUCTION

Several workers have postulated that the toxicity of oxine to microorganisms may be due to its ability to form chelate complexes with essential trace metals, which are then rendered unavailable for metabolic processes (Zentmyer 1943; Albert 1944). This view was supported by further work when Zentmyer (1944) demonstrated that oxine inhibition could be reversed by Zn, and Albert *et al.* (1947) found strong positive correlation between bacteriostatic activity and chelating power in a series of oxines, and demonstrated reversal of oxine inhibition by Co for Gram-positive bacteria, and by Zn and Fe for Gram-negative bacteria. Gale (1949) found that oxine inhibited glutamic acid assimilation in *Staphylococcus aureus*, but the addition of Mn, Co, and Fe annulled this.

That the toxicity of oxine was not entirely due to its ability to render essential metals unavailable was suggested by Mason (1948) who found that Cu oxinate was more toxic against some phytopathogenic fungi than was oxine itself. The present paper supports this view, as it has been found that in the absence of Cu, oxine is not fungistatic even at relatively high concentrations. In addition to Cu, ferrous Fe also increases the fungistatic activity of oxine. Similar findings for bacteria have recently been made by Rubbo, Albert, and Gibson (1950). No reversal of inhibition by high concentrations of Co, Zn, Fe, Cu, or Mn was obtained, but slight reversal occurred with large amounts of Mo.

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II. MATERIALS AND METHODS

(i) *Test Organisms*.—Most of the work was carried out using *Aspergillus niger*, strain 543R, but several others, viz. 540 and Mulder, gave similar results. Stock cultures were grown in tubes of liquid medium freed from trace elements as described below. Inoculum was prepared by suspending one loopful of spores in 10 ml. of twice glass-distilled water, and seeding each flask with one drop from a sterile pipette previously cleaned with aqua regia.

(ii) *Medium*.—A nutrient solution of the following composition was used: sucrose, 50 g.; KNO_3 , 5 g.; K_2HPO_4 , 2.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g.; distilled H_2O , 1000 ml. The following optimum amounts of trace elements were added to one litre of medium: Fe (ferrous), 200 μg .; Zn, 180 μg .; Cu (cupric), 40 μg .; Mn (manganous), 20 μg .; Mo (molybdate), 10 μg .; and B (borate), 10 μg .

(iii) *Removal of Trace Metals*.—As the amount of trace metals present is a critical factor in oxine inhibition, all glassware was cleaned by immersion in aqua regia overnight, followed by three rinses with water from a tinned copper still and three with double glass-distilled water.

The medium was freed from Fe, Zn, Cu, and Mn by repeated extraction with an 0.5 per cent. solution of oxine in redistilled carbon tetrachloride at pH 9, and with a solution of purified diphenylthiocarbazone (dithizone) in carbon tetrachloride at pH 6. Excess chelating agents were removed by several shakings with redistilled carbon tetrachloride at pH 9, 8, 6, and 4. In all cases the pH was adjusted either with glass-distilled HCl or NH_4OH . The degree of deficiency of various trace metals obtainable in this medium at pH 7.0 was determined by growing *A. niger* (543R) in flasks containing 25 ml. of purified medium with optimum amounts of all essential trace elements added except the one under test.

After seven days' incubation at 30°C., the mycelium from each flask was harvested, dried, and weighed. In medium with all trace elements provided, the average weight of mycelium was 0.31 g., while with deficiencies of Fe, Zn, Cu, and Mn, average weights of 0.01, 0.00(5), 0.16, and 0.19 g. respectively were obtained.

With Fe deficiency, the mycelial mat was thin, white, with a few small black spore heads; with Zn deficiency the mat was fragile and produced a few large black sporangia; in Cu-deficient medium the growth was fairly poor, white, and devoid of spores; while with Mn deficiency no mat was formed, only isolated floating papery colonies resembling puffed wheat.

(iv) *Tests for Toxicity*.—The ability of oxine to inhibit fungal growth was estimated by the dry weights of mycelium obtained after seven days' incubation at 30°C. in 25 ml. of purified medium to which known amounts of trace metals and oxine had been added. Conical Pyrex flasks of 100 ml. capacity covered with inverted beakers were used in duplicate or triplicate throughout the work. While work was in progress, it was found that the pH of the medium influenced the toxicity of oxine, and that below pH 5.0, there was no toxic effect up to a

concentration of oxine of 12 mg./l. In cases of extreme inhibition, there was either no growth at all or insufficient to reduce pH appreciably. Since the medium was poorly buffered and as *A. niger* produces large amounts of acid, daily examinations of the cultures sometimes revealed slight recovery if enough mycelium developed to lower the pH sufficiently.

III. EXPERIMENTAL RESULTS

(a) Effect of Cu on Oxine Toxicity

While purifying media from heavy metals, it was observed that residual traces of oxine were fungistatic except in the absence of Cu. Therefore an experiment was designed to determine the interactions of Cu and oxine by growing *A. niger* (543R) in purified medium at pH 6 and 7 containing various combinations ranging from Cu, 0 to 60 μ g., and oxine, 0 to 12 mg./l., together with optimum amounts of other essential trace elements. The results are set out in Table 1 and the type of growth obtained for certain treatments is shown in Plate 1.

TABLE 1
EFFECT OF COPPER ON TOXICITY OF OXINE TO *ASPERGILLUS NIGER*, 543R

Conc. of Cu		Conc. of Oxine*		Mean Wt. of Mycelium from 25 ml. Medium (g.)	
(μ g./l.)	Molarity	(mg./l.)	Molarity	pH 6	pH 7
0	0	0	0	0.17	0.16
0	0	2	1.38×10^{-5}	0.17	0.19
0	0	4	2.76×10^{-5}	0.17	0.19
0	0	8	5.52×10^{-5}	0.10	0.10
0	0	12	8.27×10^{-5}	0.00	0.00
20	3.14×10^{-7}	0	0	0.25	0.30
20	3.14×10^{-7}	0.5	3.45×10^{-6}	0.24	0.23
20	3.14×10^{-7}	0.75	4.6×10^{-6}	0.19	0.16
20	3.14×10^{-7}	1	6.90×10^{-6}	0.03	0.01
20	3.14×10^{-7}	1.25	8.62×10^{-6}	0.00 (3)	0.00
20	3.14×10^{-7}	1.5	10.3×10^{-6}	0.00	0.00
40	6.29×10^{-7}	0	0	0.29	0.31
40	6.29×10^{-7}	0.25	1.72×10^{-6}	0.24	0.00
40	6.29×10^{-7}	0.5	3.45×10^{-6}	0.24	0.00
40	6.29×10^{-7}	0.75	4.6×10^{-6}	0.00	0.00
60	9.43×10^{-7}	0	0	0.27	0.32
60	9.43×10^{-7}	0.25	1.72×10^{-6}	0.01	0.00
60	9.43×10^{-7}	0.5	3.45×10^{-6}	0.00	0.00

* One molecule of oxine combines with two atoms of divalent metal.

In the absence of Cu at both pH 6 and 7 there was no inhibition of growth at concentrations of oxine up to 4 mg., marked reduction of growth at 8 mg./l., and no mycelium at 12 mg./l. At a concentration of 20 μ g. Cu/l., growth ceased at pH 6 in the presence of 1.5 mg. oxine/l., while at pH 7, it ceased at

1.25 mg. oxine/l. With 40 μ g. Cu/l., no growth appeared at pH 6 and 7 in the presence of 0.75 and 0.25 mg. oxine/l. respectively. Finally at 60 μ g. Cu/l., growth failed at pH 6 and 7 at 0.5 and 0.25 mg. oxine/l. respectively. In the absence of oxine, Cu showed no toxicity even at the highest concentrations.

These results indicate that the higher the concentration of added Cu, the greater the toxicity of oxine. This is more marked at pH 7 than at pH 6. These facts suggest that Cu oxinate is the toxic substance.

(b) Effect of pH on Oxine Toxicity

The difference in behaviour of oxine in the presence of Cu at pH 6 and 7 suggested an experiment to test the effect of varying the pH from 2 to 10 in the presence and absence of Cu. The results are summarized in Table 2.

TABLE 2
INFLUENCE OF pH ON TOXICITY OF OXINE TO *ASPERGILLUS NIGER*, 543R IN THE PRESENCE AND ABSENCE OF COPPER

Conc. of Cu (μ g./l.) Molarity		Conc. of Oxine* (mg./l.) Molarity		Mean Wt. of Mycelium from 25 ml. Medium (g.)									
				pH 2	pH 3.5	pH 4	pH 5	pH 6	pH 7	pH 7.5	pH 8	pH 8.5 and 10	
0	0	0	0	0.15	0.16	0.18	0.18	0.17	0.16	0.16	0.13	0.00	
0	0	3	2.7	0.20	0.17	0.20	0.18	0.17	0.19	0.12	0.07	0.00	
			$\times 10^{-5}$										
40	6.29	0	0	0.25	0.31	0.31	0.31	0.29	0.31	0.30	0.24	0.00	
	$\times 10^{-7}$												
40	6.29	3	2.7	0.23	0.31	0.27	0.20	0.00	0.00	0.00	0.00	0.00	
	$\times 10^{-7}$		$\times 10^{-5}$										

* One molecule of oxine combines with two atoms of divalent metal.

When both Cu and oxine were omitted, typical Cu-deficient mycelium was obtained and the weight did not vary significantly over the pH range from 2 to 7.5; there was a slight decrease at pH 8 and no growth at higher pH values. In the absence of Cu and in the presence of oxine growth remained unchanged from pH 2 to 7; slight reduction occurred at pH 7.5, and no growth above pH 8.

At the optimum concentration of Cu of 40 μ g./l. with no oxine added, normal weights of mycelium were obtained from pH 3.5 to 7.5; a slight reduction occurred at either end of this range, viz. pH 2 and 8, while no mycelium developed above pH 8. At the optimum level of Cu when oxine was added there was normal growth from pH 2 to 5, slight toxicity at pH 5, and complete inhibition at higher pH values. This indicates that the toxicity of oxine is greatly reduced at low pH even in the presence of Cu. It suggests that toxic Cu oxinate is decomposed or is rendered less harmful by acidity.

(c) Effect of Other Metals on Oxine Toxicity

It was thought that other trace metals might also increase the toxicity of oxine to *A. niger* (543R), so an experiment was set up in the presence and absence of oxine, using media at pH 7 deficient in Fe, in Zn, in Mn, or partially deficient in Mo.

It was found that, in the absence of Fe, the weights of mycelium with 0, 0.75, and 2 mg. oxine/l. were 0.05, 0.04, and nil respectively. In the presence of 200 μ g. Fe/l. and the absence of oxine the mycelial weight was 0.31 g., but when oxine was present in either concentration, no growth was obtained. This indicates that Fe aggravates the fungistatic action of oxine, but to a lesser degree than Cu. This suggests that Fe oxinate is less fungistatic than Cu oxinate. The effect is more noticeable in the absence of Cu than of Fe because the weight of mycelium in Cu-deficient medium is still quite appreciable. Unlike Cu and Fe, deficiencies of Zn, Mn, and Mo did not lessen the toxicity of oxine.

Further work was done at pH 6 on the effect of different concentrations of ferrous Fe on the toxicity of oxine at various levels (Table 3).

TABLE 3
EFFECT OF FERROUS IRON ON TOXICITY OF OXINE TO *ASPERGILLUS NIGER*, 543R

Conc. of Oxine*		Mean Wt. of Mycelium from 25 ml. Medium at Different Conc. of Fe (g.)		
		200	700	1200
		μ g./l.	μ g./l.	μ g./l.
(mg./l.)	Molarity			
0	0	0.29	0.27	0.27
0.25	1.72×10^{-6}	0.24	0.21	0.18
0.5	3.45×10^{-6}	0.24	0.00	0.00
0.75	4.6×10^{-6}	0.00	0.00	0.00

* One molecule of oxine combines with two atoms of divalent metal.

It is apparent that, in the absence of oxine, normal growth occurred even up to the highest concentration of Fe used. At 0.25 mg. oxine/l. growth was almost normal at 200 μ g. Fe/l. but declined slightly at higher levels of Fe. At 0.5 and 0.75 mg. oxine/l., growth ceased at 700 and 200 μ g. Fe/l. respectively. This shows that the higher the concentration of added Fe the greater the toxicity of oxine.

Until now the work had been concerned with the effect of trace metals in increasing the toxicity of oxine. Since other authors had shown that certain trace metals could reverse oxine inhibition this was now tested by adding Zn, Mn, Fe, Cu, Co, and Mo singly to a purified medium containing optimum amounts of essential elements. The concentration of additional trace metals ranged from the optimum amount up to 1000 μ g./l., i.e. amounts greater than were necessary to combine with the oxine that was added at the level of 0.7 mg./l.

In the absence of oxine, in no instance were these amounts of added trace elements inhibitory. In the presence of oxine the addition of Zn, Mn, Fe, or Cu did not lessen toxicity since no growth occurred, but in the presence of 1000 μ g. Mo slight growth appeared after three days' incubation. This suggests that Mo is the only trace element capable of reversing the fungistatic activity of oxine.

(d) Fungistatic Activity of Other Chelators

It was of interest to know whether chelators other than oxine possessed fungistatic activity, and whether or not this was influenced by the Cu content of the medium. Consequently *A. niger* (543R) was grown in purified medium at pH 7 containing 0 or 40 μ g. Cu/l., together with optimum amounts of other essential elements, after the addition of various chelators separately in the following concentrations: sodium diethyldithiocarbamate, cupferron, or *o*-phenanthroline from 0 to 10 mg./l.; 4-methyl-1:2-dimercaptobenzene (dithiol) or diphenylthiocarbazone (dithizone) from 0 to 20 mg./l.; 1-hydroxyphenazine or 6-hydroxy-*m*-phenanthroline from 0 to 3 mg./l.

It was found that sodium diethyldithiocarbamate or cupferron was not toxic at the highest levels, either in the presence or absence of Cu. At the highest concentration of *o*-phenanthroline, growth was only slightly inhibited in the presence of Cu, but totally suppressed in its absence. In this case Cu reversed the toxic nature of this chelator. With dithiol or dithizone (20 mg./l.), the fungus was not inhibited in Cu-deficient medium, but growth was retarded somewhat when Cu was supplied. 1-Hydroxyphenazine and 6-hydroxy-*m*-phenanthroline, which closely resemble 8-hydroxyquinoline in possessing chelating groups in the same positions, behaved somewhat like oxine by partially inhibiting the growth of the fungus in the presence of Cu, but not in its absence.

IV. DISCUSSION

It has already been mentioned that a number of authors consider that the toxicity of oxine might be due to its ability to chelate with trace metals, either in the medium or more probably on or inside the cells, and thus deprive organisms of metals essential for metabolism. This argument gained support from the observation that high concentrations of certain metal cations can reverse oxine inhibition.

While this may be partly true for *A. niger*, it is not the whole explanation, since the addition of optimal amounts of Cu and Fe greatly increases the fungistatic action while their removal lessens it. This suggests that Cu and Fe oxinates are more toxic than free oxine, which at pH 7 occurs almost entirely as the neutral molecule (Albert and Magrath 1947). It is difficult to see why they should be so toxic except that at this pH, Cu and Fe oxinates are much more soluble in organic solvents than in water, whereas with oxine this partition is not so marked, and tests showed that coloured metal complexes were readily adsorbed from water by *A. niger* mycelium, which possesses a lipid-protein cell membrane. It seems likely that neutral, relatively inert molecules like Cu and Fe oxinates are adsorbed onto vital cell interfaces, not by ionic or covalent linkages, but rather by van der Waal forces. Since the degree of adsorption and bacteriostatic activity of heterocyclic bases are partly determined by the area and flatness of the molecule (Albert, Rubbo, and Burvill 1949), it is conceivable that metal oxinates, which are flat molecules with over twice the area of the oxine molecule, owe part of their high toxicity to their large surface

area. If this were true, then it is difficult to understand why other metal oxinates with similar flat molecular areas and with stability constants between those of Cu and Fe oxinates, e.g. those of Co and Zn (Maley and Mellor 1949) are not equally effective fungistats at pH 7.

Assuming that adsorption has occurred, and that the toxic action takes place on some cell surface, it is still more difficult to speculate how a relatively stable molecule like Cu oxinate acts fungistatically. It seems unlikely that such small amounts of Cu oxinate could adversely poise the redox potential of the fungus. There is a possibility that Cu oxinate might act as a reversible H carrier and promote complete oxidation of some essential metabolite (Albert and Falk 1949). If Cu oxinate functions by immobilizing other metallic cations essential for the mould, then probably this could be done only after displacing the Cu. A cell component, with chelating properties and having a stronger avidity for Cu than oxine, might do this by donating H, e.g. substances with thiol groups (Bernheim and Bernheim 1939). The oxine cations thereby produced might then become the intra-cellular poison. The Cu in Cu oxinate might also be displaced by other metal cations capable of forming more stable complexes, e.g. Pd, or by the mass action effect of abnormally high concentrations of cations with lower stability constants, e.g. Co, Zn, and Mn (Maley and Mellor 1949). This does not seem to happen except with Mo. Therefore the mode of fungistatic action of Cu oxinate (or of Fe oxinate) remains unknown.

The observation that the toxicity of Cu oxinate is nullified by lowering the pH to 4 suggests that at this acidity most of the metal complex has been decomposed, giving a mixture of Cu^{++} ions, oxine cation, and the neutral molecule. This seems reasonable as the pH for 50 per cent. dissociation of Cu oxinate is near this value. Since the pK_{NH} of oxine is 5.03 (Albert and Magrath 1947) then at pH 4 a higher concentration of oxine cation would be present than of the undissociated oxine, while at still lower pH values the amount of oxine cation would rise still further and the amounts of neutral oxine and Cu oxinate would become almost negligible. It has been shown that the bacteriostatic activity of heterocyclic bases is directly proportional to their degree of cationic ionization (Albert, Rubbo, and Burvill 1949). It is therefore surprising that the toxicity of oxine does not again increase as the pH approaches 2.

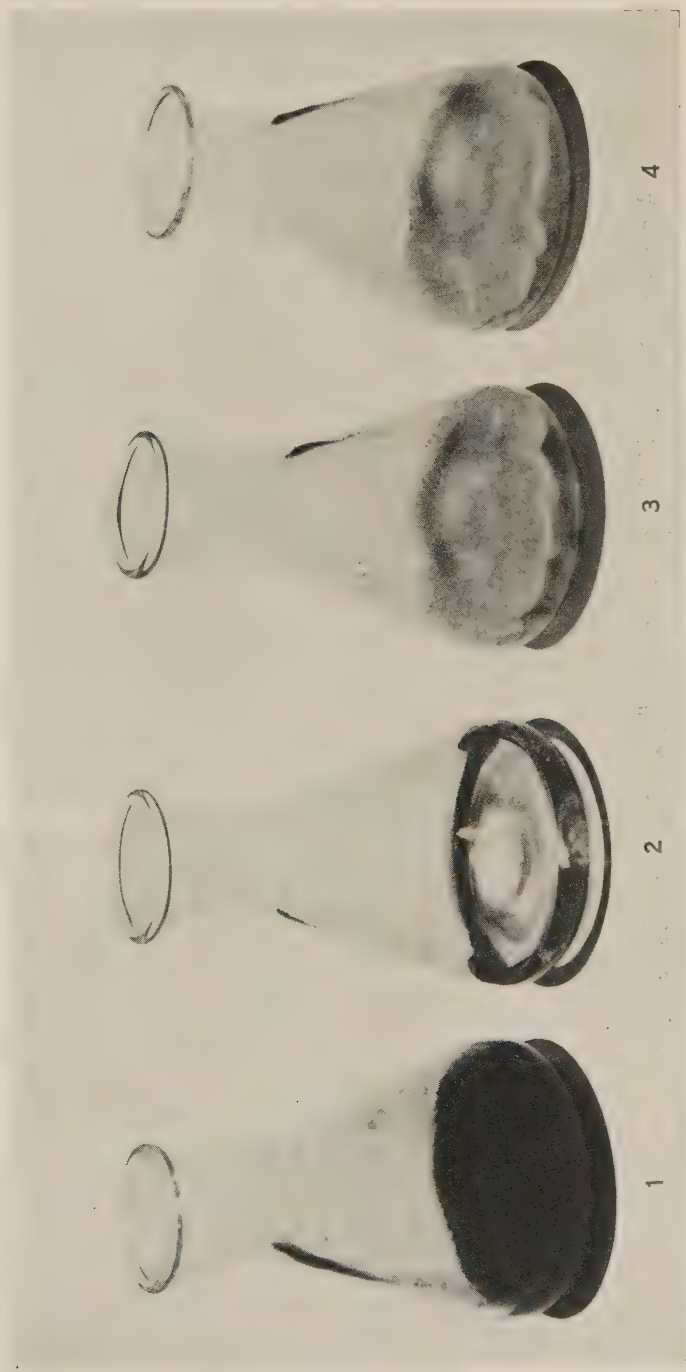
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VI. REFERENCES

- ALBERT, A. (1944).—Kationic chemotherapy, with special reference to the acridines. *Med. J. Aust.* 1: 245.
- ALBERT, A., and FALK, J. E. (1949).—The formation of hydrogen carriers by haematin-catalysed peroxidations. 1. Hydrogen carriers from certain acridine and quinoline compounds. *Biochem. J.* 44: 129.
- ALBERT, A., and MAGRATH, D. (1947).—The choice of a chelating agent for inactivating trace metals. 2. Derivatives of oxine (8-hydroxyquinoline). *Biochem. J.* 41: 534.
- ALBERT, A., RUBBO, S. D., and BURVILL, MARGARET, I. (1949).—The influence of chemical constitution on antibacterial activity. IV. A survey of heterocyclic bases, with special reference to benzquinolines, phenanthridines, benzacridines, quinolines, and pyridines. *Brit. J. Exp. Path.* 30: 159.
- ALBERT, A., RUBBO, S. D., GOLDACRE, R. J., and BALFOUR, B. G. (1947).—The influence of chemical constitution on antibacterial activity. III. A study of 8-hydroxyquinoline (oxine) and related compounds. *Brit. J. Exp. Path.* 28: 69.
- BERNHEIM, F., and BERNHEIM, N. L. C. (1939).—The effects of various metals and metal complexes on the oxidation of sulfhydryl groups. *Cold Spr. Harb. Symp. Quant. Biol.* 7: 174.
- GALE, E. F. (1949).—The assimilation of amino-acids by bacteria. 8. Trace metals in glutamic acid assimilation and their inactivation by 8-hydroxyquinoline. *J. Gen. Microbiol.* 3: 369.
- MALEY, L. E., and MELLOR, D. P. (1949).—The relative stability of internal metal complexes. II. Metal derivatives of 8-hydroxyquinoline 5-sulphonic acid and a series of monocarboxylic mono- α -amino acids including histidine. *Aust. J. Sci. Res. A* 2: 579.
- MASON, C. L. (1948).—A study of the fungicidal action of 8-quinolinol and some of its derivatives. *Phytopathology* 38: 740.
- RUBBO, S. D., ALBERT, A., and GIBSON, MARGARET I. (1950).—The influence of chemical constitution on antibacterial activity. V. The antibacterial action of 8-hydroxyquinoline (oxine). *Brit. J. Exp. Path.* 31: 425.
- ZENTMYER, G. A. (1943).—Mechanism of action of 8-hydroxyquinoline. *Phytopathology* 33: 1121.
- ZENTMYER, G. A. (1944).—Inhibition of metal catalysis as a fungistatic mechanism. *Science* 100: 294.

FUNGISTATIC ACTION OF 8-HYDROXYQUINOLINE



Effect of two concentrations of Cu on growth of *Aspergillus niger*, 543R, in the presence and absence of oxine. L. to R.: 1. Cu, 40 $\mu\text{g./l.}$; oxine, nil; 2. Cu, 40 $\mu\text{g./l.}$; oxine, 0.75 mg./l. 3. Cu, 20 $\mu\text{g./l.}$; oxine, nil. 4. Cu, 20 $\mu\text{g./l.}$; oxine, 0.75 mg./l.

VOLATILE PRODUCTS OF APPLES

I. IDENTIFICATION OF ACIDS AND ALCOHOLS

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[Manuscript received September 19, 1950]

Summary

The volatile substances given off to the air by Granny Smith apples at 20°C. were condensed at low temperature. The acids obtained on saponification were found to be virtually free from carbonyl, hydroxy, or unsaturated groups. By conversion to hydroxamic acids followed by chromatographic separation on paper they were identified as formic, acetic, propionic, butyric (probably normal), valeric, and caproic acids. All these acids were shown to be present in the esterified form. Formic and acetic acids were shown to occur in both the free and esterified forms. All these acids do not necessarily occur in every sample of volatiles. For instance, formic acid has been found in some samples but not in others.

The alcohols obtained on saponification were found to be predominantly primary and saturated and the major constituents were found to be methanol, ethanol, and *n*-propanol. Ethanol and *n*-propanol were identified by paper chromatography after conversion to hydroxamic acids and methanol by a specific colour test.

I. INTRODUCTION

There is evidence that the volatile substances given off to the atmosphere by apples are responsible for superficial scald, a functional disorder of cold-stored fruit (Brooks, Cooley, and Fisher 1919). A systematic study of these volatile substances is being made and this paper describes methods for the identification of the acids and alcohols present in free and esterified forms.

Power and Chesnut (1920) identified methyl, ethyl, and amyl esters of formic, acetic, caproic, and caprylic acids in the steam distillate of the parings of certain apple varieties.

White (1950) found esters of formic, acetic, propionic, butyric, and caproic acids in a concentrate of volatiles distilled from apple juice. Methanol, ethanol, 2-propanol, and butanol were identified as the alcoholic components of the esters. A high proportion of the volatile concentrate was composed of free alcohols which were identified as methanol, ethanol, *n*-propanol, 2-propanol, butanol, isobutanol, *d*-2-methyl-1-butanol, and hexyl alcohol. The carbonyl compounds comprised acetaldehyde, acetone, caproaldehyde, and 2-hexenal.

Of the substances given off to the atmosphere by fresh apples at ordinary temperatures, carbon dioxide, ethylene (Gane 1935), and acetaldehyde (Power and Chesnut 1920) have been identified. Walls (1942) has given evidence for the presence of esters of amyl alcohol and of formic and acetic acids among

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the volatile substances from whole Lane's Prince Albert apples after absorption on granular calcium chloride.

In the present work the volatile substances given off to the air by Granny Smith apples at 20°C. were collected by condensation at low temperatures and the constituent acids, free and esterified, and alcohols, free and esterified, were identified.

II. FREE AND ESTERIFIED ACIDS

(a) Collection and Preparation of Sample

Air, purified by combustion of organic matter and absorption of carbon dioxide, was passed over 10 kg. of Granny Smith apples at 20°C. The volatiles were condensed for two to five days in a spiral absorber cooled in liquid oxygen. The esters were hydrolysed by addition of 0.2N sodium hydroxide. The alcohols were removed by distillation from the alkaline solution, which was then acidified and the acids distilled. A preliminary test indicated that acids up to decanoic would be recovered in this procedure. The acid distillate was brought to pH 8 with sodium hydroxide and evaporated to dryness. The sodium salts of all the acids, both free and esterified, were thus obtained.

Tests were first made to determine whether these acids were predominantly simple saturated fatty acids or whether they contained carbonyl, hydroxy, or unsaturated groupings.

(b) Test for Carbonyl Group

The sensitive method of Friedman and Haugen (1943) for the estimation of carbonyl compounds was used. This method measures the colour produced by reaction with 2,4-dinitrophenylhydrazine in acid solution followed by the addition of sodium hydroxide. The colorimeter readings were converted to equivalents of carbonyl compounds, using pyruvic acid as standard. The mean molecular weight of the mixed sodium salts was assumed to be equal to that of sodium butyrate. This assumption was justified by the identification of the individual acids. The tests indicated not more than one carbonyl group in 1400 moles of mixed acids.

(c) Test for Hydroxyl Group

The hydroxy content of the mixed sodium salts was estimated by acetylation. The mixed sodium salts (20 mg.) and acetyl chloride (15 drops) were allowed to stand for 15 minutes. Water (approximately 2 ml.) was added and allowed to stand for 30 minutes to decompose the excess acetyl chloride. The acetyl derivatives were separated by extraction with ether. The ether extract was treated with hydroxylamine in alkaline solution to give acethydroxamic acid, which was estimated colorimetrically (Thompson 1950). Lactic acid was used to standardize the method. The test indicated not more than one hydroxy group in 165 moles of mixed acids. The hydroxy content may be appreciably less, as small amounts of anhydrides may resist hydrolysis and be included in the determination.

(d) *Test for Unsaturation*

With the small amount of material available, the only satisfactory method of determining unsaturation was found to be hydrogenation with a palladium catalyst in a Warburg flask with manometric estimation of the hydrogen absorbed (Milton and Waters 1949). The figure obtained indicated a ratio of one mole of hydrogen (corresponding to one double bond) to 385 moles of mixed acids.

(e) *Identification as Hydroxamic Acids*

The sample of sodium salts was converted to a mixture of hydroxamic acids in the following manner:

Thionyl chloride (0.2 ml.) was added to the dry sodium salts (10 mg.) and allowed to stand for 15 minutes. Absolute ethyl alcohol (5 ml.) was added and allowed to stand for a further 30 minutes. Water was added and, after allowing several minutes for the decomposition of the thionyl chloride, the esters were extracted from the solution with ether (30 ml.). The ether solution was dried over calcium chloride and neutralized with alcoholic sodium hydroxide. The hydroxamic acids were formed by reaction with hydroxylamine as described in an earlier paper (Thompson 1950). The efficiency of conversion of carboxylic to hydroxamic acid was found to be approximately 70 per cent. with *n*-butyric acid.

The chromatographic separation of the hydroxamic acids was carried out as described previously (Thompson 1951). By comparing the R_F values with those of known hydroxamic acids run on the same paper the acids derived from apples were identified (Plate 1 and Plate 2, Fig. 1). The acids are generally described by the older names which only indicate number of carbon atoms, as in most cases it was impossible to distinguish between isomers.

The relative proportions of the acids varied somewhat with different samples. A sample examined in 1949 was shown to contain acetic, propionic, butyric, valeric, and caproic acids (Plate 1*d*). The chromatogram was obtained with benzene-acetic acid. This test did not prove the absence of formic acid, as in 1949 a solution of ferric perchlorate containing excess perchloric acid was used to develop the chromatograms. Under these conditions formhydroxamic acid is unstable. However, by means of a separate colorimetric test based on reduction to formaldehyde (Grant 1943) and reaction with chromotropic acid (MacFadyen 1945), it was shown that the proportion of formic acid was negligible.

A sample from less mature apples examined in 1950 was found to contain formic and acetic as predominant acids (Plate 2, Fig. 1*b*). A small amount of propionic acid and only traces of higher acids were present. Butanol-acetic acid was used for this test. The spots were developed with ferric chloride to which formhydroxamic acid is quite stable. The chromotropic acid test for formic acid was strongly positive.

III. ESTERIFIED ACIDS

For identification of esterified acids the volatile substances from apples were more conveniently condensed in ether (30 ml.) in a spiral absorber cooled in a mixture of solid carbon dioxide and ethanol. The efficiency of collection of esters in this absorber was about 95 per cent. The ether layer was separated from the water layer and dried. The esters were converted directly to hydroxamic acids by reaction with hydroxylamine (Thompson 1950), and identified by paper chromatography as already described.

For each sample of apples examined all the acids obtained on hydrolysis were represented in the esters. The esterified acids from the 1949 sample of apples were found to include acetic (trace), propionic, butyric (major constituent), valeric (trace), and caproic acids. This is shown in Plate 3, Figure 1 (caprylic alcohol-oxalic acid) and Plate 1*b* (benzene-acetic acid). In Plate 3, Figure 2 (benzene-acetic acid), the absence of higher acids is demonstrated. Storage of these apples at 0°C. for various periods up to 30 weeks made no significant difference to the composition of the esterified acids.

TABLE 1
R_F VALUES OF BUTYRO-HYDROXAMIC ACIDS FROM ESTERS

Paper	Test Solutions		
	Normal Butyrate	"Apple" Butyrate	<i>iso</i> Butyrate
1	0.127	0.125	0.133
	0.148	0.143	0.148
	0.143	0.142	0.160
	0.134	0.140	0.168
2	0.136	0.152	0.162
	0.150	0.151	0.148
	0.137	0.134	0.145
	0.104	0.117	0.136
3	0.112	0.120	0.135
	0.122	0.132	0.132
	0.127	0.125	0.158
	0.125	0.125	0.147

From the less mature apples examined in 1950 only formic acid and acetic acid were identified amongst the esterified acids. Butanol-acetic acid was used as solvent mixture (Plate 2, Fig. 1*a*).

An attempt was made to determine whether the butyrate present was normal or *isobutyrate*. It was possible, by paper chromatography with benzene and acetic acid, to effect a small separation of normal and *isobutyro*-hydroxamic acids. By statistical treatment of the R_F values (Table 1) of the two isomers and of the butyrohoxamic acid derived from apples, it was

shown that the probability of the "apple" butyrate being *isobutyrate* is less than 0.001, whereas the probability of it being normal butyrate is greater than 0.2 (Table 2).

TABLE 2
ANALYSIS OF VARIANCE OF DATA IN TABLE 1

Source of Variation	D.F.	Mean Squares	Ratio
Between solutions (<i>n</i> -butyrate, "apple" butyrate and <i>iso</i> -butyrate) (S)	2	0.000950	16.10***
Between papers (P)	2	0.000550	9.32**
S \times P	4	0.000024	N.S.
Between replicates within papers (R)	9	0.000337	5.71***
R \times S	18	0.000059	
Total	35		
"Apple" butyrate v. <i>isobutyrate</i>	1	0.001080	18.31***
<i>n</i> -Butyrate v. (mean of "apple" and <i>isobutyrate</i>)	1	0.000820	13.90**
"Apple" butyrate v. <i>n</i> -butyrate	1	0.000070	1.19 (N.S.)
<i>iso</i> Butyrate v. (mean of "apple" butyrate and <i>n</i> -butyrate)	1	0.001830	31.02***

N.S. = not significant; * = significant at 5 per cent. level; ** = significant at 1 per cent. level; *** = significant at 0.1 per cent. level.

IV. FREE ACIDS

The volatile substances from apples were collected in ether cooled in a solid carbon dioxide-alcohol bath. The ether solution, with a similar volume of water, was neutralized with 0.1N sodium hydroxide using phenolphthalein as indicator and shaking vigorously throughout. The aqueous layer was separated and evaporated to dryness. The sodium salts of the free apple acids were converted to hydroxamic acids as described in Section II and separated by paper chromatography. Only the free acids from the less mature apples in 1950 were examined by this procedure. Using phenol or butanol-acetic acid (Plate 2, Fig. 2), formic and acetic acids were identified but no higher acids. Formic acid was also identified by the chromotropic acid test.

It had previously been shown that the esterified acids from these apples were mainly formic and acetic. It appears, therefore, that formic and acetic acids, when present, occur free as well as esterified. The higher acids predominated in the esterified acids from the more mature apples in 1949, but unfortunately no attempt was made to identify the free acids from these apples. Hence there is no evidence that the higher acids occur free but the possibility is not excluded.

V. FREE AND ESTERIFIED ALCOHOLS

(a) Preparation of Solution

The distillate from the alkaline solution of saponified volatiles collected in 1949 (see Section II (a)) was used for the identification of alcohols. A micro-hydrogenation test indicated negligible unsaturation, amounting to approximately one double bond in 600 moles. This calculation was based on the weight of sodium salts obtained on oxidation, assuming an average molecular weight equal to sodium acetate.

A colorimetric test (Friedman and Haugen 1943) showed the presence of trace amounts of carbonyl compounds. The distillate was therefore treated with the 2,4-dinitrophenylhydrazine solution and distilled under reduced pressure, with ice water in the condenser, to obtain a distillate free from carbonyl compounds.

(b) Test for Secondary Alcohols

An aliquot of the carbonyl-free distillate was oxidized with dilute chromic acid and again distilled. The distillate of oxidized products was free from carbonyl compounds, indicating the absence of secondary alcohols before oxidation.

(c) Identification as Hydroxamic Acids

The carbonyl-free solution of primary alcohols was oxidized with chromic acid and the acids distilled off. The distillate was brought to pH 8 with sodium hydroxide and evaporated to dryness. The sodium salts of the fatty acids were converted by hydroxamic acids by the method previously described (Section II (e)).

Hydroxamic acids containing two, three, and six carbon atoms (the last only in traces) were identified on the chromatogram (Plate 1c). The presence of ethanol and *n*-propanol and a trace of six-carbon alcohol in the original solution was thus established. The use of ferric perchlorate-perchloric acid solution as developer prevented the detection of formhydroxamic acid and hence methanol in the solution of alcohols.

However, the presence of methanol was shown by a specific test on a solution of apple alcohols. After oxidation with permanganate (Feigl 1943) the solution gave a positive test for formaldehyde with chromotropic acid. No formaldehyde was detected before oxidation.

VI. DISCUSSION

The author has found saturated fatty acids containing one, two, three, four, five, and six carbon atoms and saturated alcohols containing one, two, three, and six carbon atoms in the volatiles from fresh apples. It is probable that each acid and alcohol can occur both in the free and esterified form. All the acids have been found as esters, and formic and acetic acids have also been found free. The higher acids—free and esterified—were virtually absent from the only sample tested for free acids.

No other acids were found by White (1950) and Walls (1942), but an acid with eight carbon atoms was found by Power and Chesnut (1920). Walls (1942) and Power and Chesnut (1920) found amyl alcohol, and White (1950) found alcohols containing one, two, three, four, five, and six carbon atoms. Although White's work was based on the volatile fraction of apple juice, it is probable that every member of this series was present in the original fresh apples from which the juice was derived, as all except the four-carbon alcohols have been found either by Walls (1942) or the author in the volatiles from whole fresh apples.

The volatile fraction of apple juice analysed by White (1950) was found to contain 92 per cent. of free alcohols, 6 per cent. of carbonyl compounds, and only 2 per cent. of esters. The ratio of esters to free alcohols in the volatiles evolved by fresh apples would probably be higher since the esters may more readily pass through the lipoid phase of the cuticle and be preferentially evolved. Moreover, in preparation of the juice, part of the esters may be retained in the pressed residue, and there may also be subsequent loss of esters by hydrolysis.

The author's results, in conjunction with those of other workers, provide definite evidence that saturated acids and alcohols containing one, two, three, four, five, and six carbon atoms can occur in the volatiles from fresh apples and enter into a large variety of ester combinations. The relative proportions of the different components may vary from sample to sample, thus reflecting the differences in aroma and flavour associated with variety, maturity, and other factors.

The presence of homologous series of acids and alcohols with both odd and even numbers of carbon atoms is unusual among natural products. Products containing only even-numbered acids are more common, but recently Weitkamp, Smiljanic, and Rothman (1947) have demonstrated the presence of a complete series of odd-numbered fatty acids with seven to 17 carbon atoms, as well as even-numbered acids, in human hair fat.

Some comments may be made on the mechanism by which these substances are formed in the apple. Presumably each alcohol is interconvertible with the corresponding acid by oxidation and reduction. The current biochemical theories for the synthesis of fatty acids *in vivo* account only for the production of even-numbered straight chains. An important contribution to the mechanism of fatty acid synthesis has been made by the recent discovery of Stadtman and Barker (1949) that cell-free extracts of *Clostridium kluyveri* catalyse the linkage of ethanol and acetate to fatty acids of four and six carbon atoms. Both ethanol and acetate are closely related to the respiratory cycle. The linkage of odd-numbered acids or alcohols with two-carbon fragments has not been investigated but there remains the possibility of obtaining the higher odd-numbered acids by this means. A one-carbon fragment may be necessary for the initial condensation and this could be readily provided as methanol, which is always present in apple tissue as the methyl ester of pectic acid. The reduction of three-carbon intermediates of respiration to propionic acid is also possible. To obtain branched chains, linkage of fragments at other than end

groups would be necessary. This mechanism could not account for the formation of the secondary alcohol 2-propanol, which was found by White (1950) in the volatile concentrate from apple juice.

VII. ACKNOWLEDGMENTS

The author wishes to thank Dr. F. E. Huelin for his interest and advice in this work, Mr. G. Ferris for the statistical analyses, and Mr. Bruce Kennett for technical assistance. Thanks are also due to Dr. A. L. G. Rees, Mr. G. R. Hercus, and Dr. J. D. Morrison for undertaking mass spectrometric analyses of samples of apple volatiles. Their results are described in Appendix I.

VIII. REFERENCES

- BROOKS, C., COOLEY, J. S., and FISHER, D. F. (1919).—Nature and control of apple scald. *J. Agric. Res.* **18**: 211-40.
- FEIGL, F. (1943).—"Laboratory Manual of Spot Tests." pp. 193-4. (Academic Press Inc.: New York.)
- FRIEDMAN, T. E., and HAUGEN, C. E. (1943).—Pyruvic acid. II. The determination of keto acids in blood and urine. *J. Biol. Chem.* **147**: 415-42.
- GANE, R. (1935).—The formation of ethylene by plant tissues and its significance in the ripening of fruits. *J. Pomol.* **13**: 351-8.
- GRANT, W. M. (1948).—Colorimetric determination of formic acid based on reduction to formaldehyde. *Anal. Chem.* **20**: 267-9.
- MACFADYEN, D. A. (1945).—Estimation of formaldehyde in biological mixtures. *J. Biol. Chem.* **158**: 107-33.
- MILTON, R. F., and WATERS, W. A. (1949).—"Methods of Quantitative Micro-analysis." pp. 568-90. (Edward Arnold & Co.: London.)
- POWER, F. B., and CHESNUT, V. K. (1920).—The odorous constituents of apples. *J. Amer. Chem. Soc.* **42**: 1509-26.
- STADTMAN, E. R., and BARKER, H. A. (1949).—Fatty acid synthesis by enzyme preparations of *Clostridium kluyveri*. I. Preparation of cell-free extracts that catalyse the conversion of ethanol and acetate to butyrate and caproate. *J. Biol. Chem.* **180**: 1085-93.
- THOMPSON, A. R. (1950).—A colorimetric method for the determination of esters. *Aust. J. Sci. Res. A* **3**: 128-35.
- THOMPSON, A. R. (1951).—Separation of saturated mono-hydroxamic acids by partition chromatography on paper. *Aust. J. Sci. Res. B* **4**: 180-6.
- WALLS, L. P. (1942).—The nature of the volatile products from apples. *J. Pomol.* **20**: 59-67.
- WEITKAMP, A. W., SMILJANIC, A. M., and ROTHMAN, S. (1947).—The free fatty acids of human hair fat. *J. Amer. Chem. Soc.* **69**: 1936-9.
- WHITE, J. W. (1950).—Composition of a volatile fraction of apples. *Food Res.* **15**: 68-78.

APPENDIX I

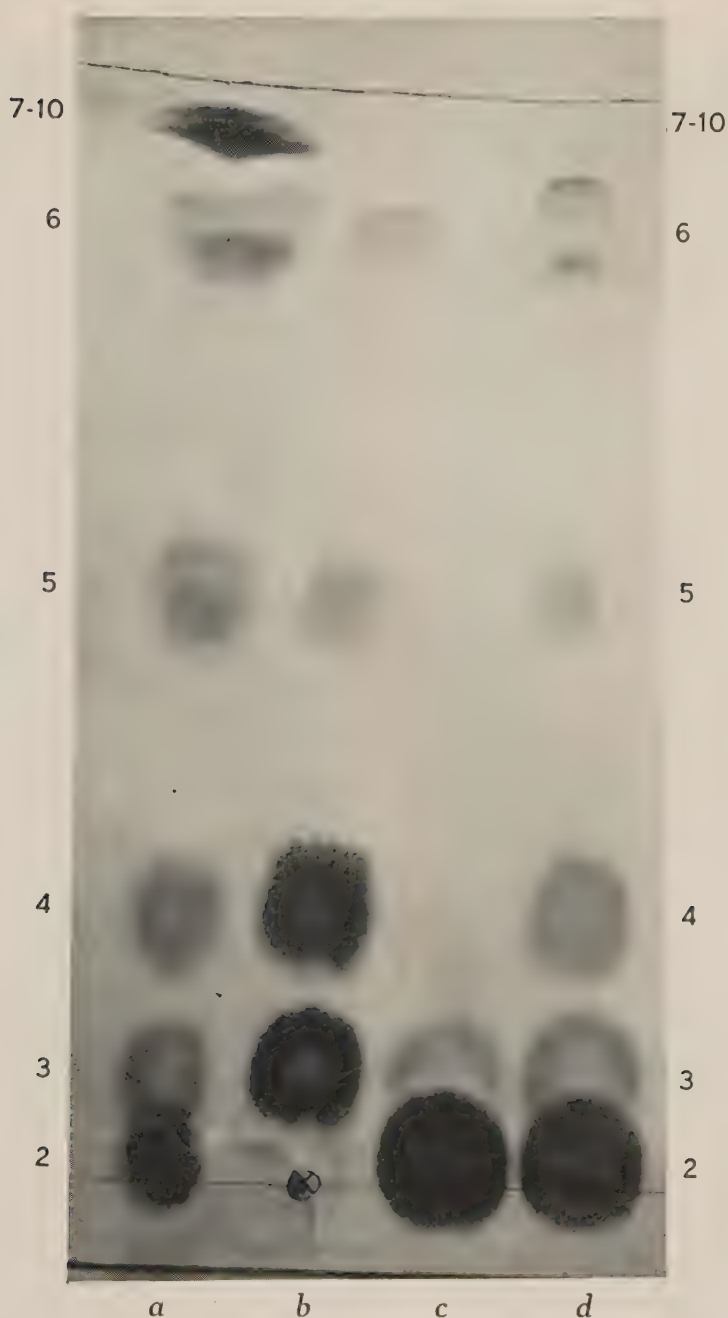
MASS SPECTROMETRIC ANALYSES

By G. R. HERCUS* and J. D. MORRISON*

In conjunction with the work reported in the above paper, a mass spectrometric examination of the apple volatile products was made, giving results in substantial agreement with those obtained above.

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VOLATILE PRODUCTS OF APPLES. I



Paper chromatogram run with benzene-acetic acid showing ferric hydroxamate spots derived from:

- (a) An artificial mixture of esters containing acids with one to ten carbon atoms;
- (b) Esterified acids from apples (1949);
- (c) Free and esterified alcohols from apples (1949);
- (d) Free and esterified acids from apples (1949). The appearance of two spots in the C6 position is due to "shadowing" (Thompson 1951).

VOLATILE PRODUCTS OF APPLES. I

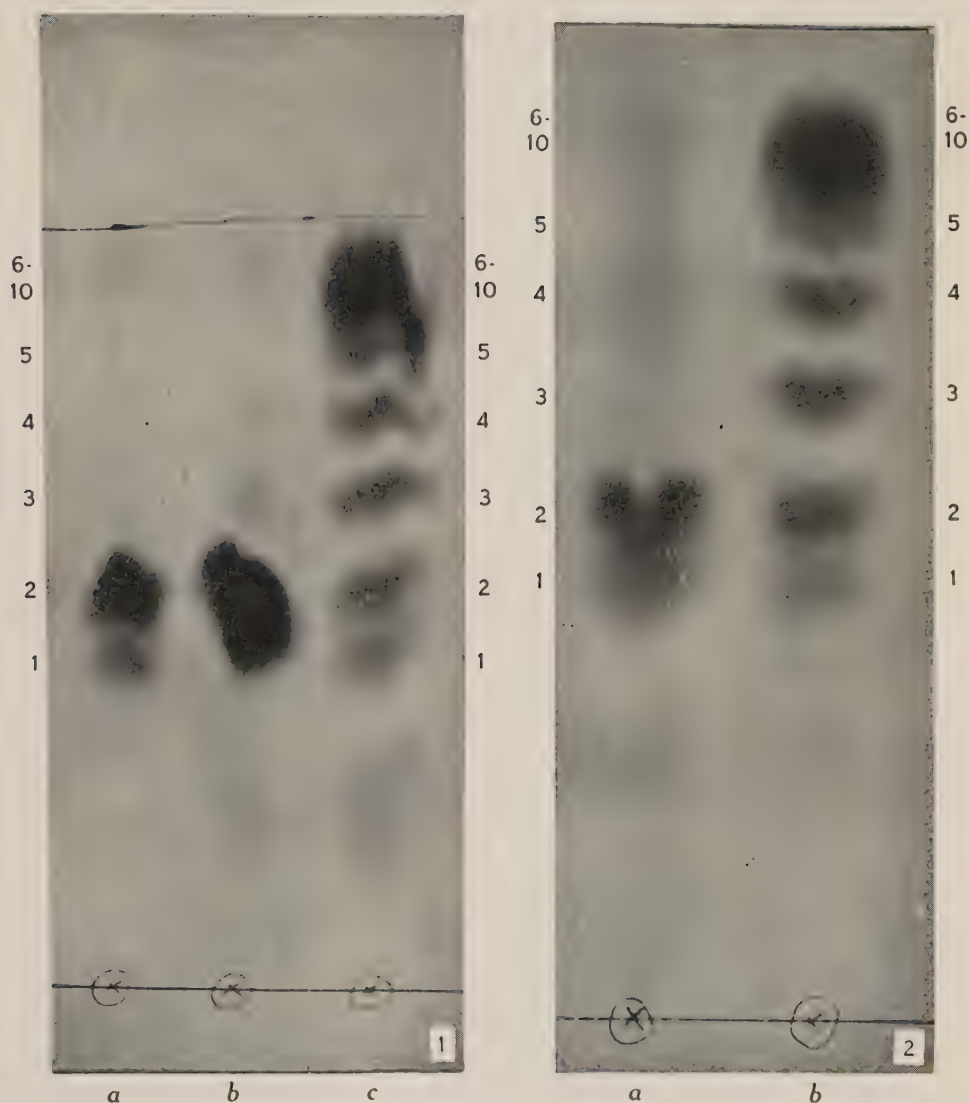


Fig. 1.—Paper chromatogram run with butanol-acetic acid showing ferric hydroxamate spots derived from:

- (a) Esterified acids from apples (1950);
- (b) Free and esterified acids from apples (1950);
- (c) An artificial mixture of esters containing acids with one to ten carbon atoms.

Fig. 2.—Paper chromatogram run with butanol-acetic acid showing ferric hydroxamate spots derived from:

- (a) Free acids from apples (1950);
- (b) An artificial mixture of esters containing acids with one to ten carbon atoms.

VOLATILE PRODUCTS OF APPLES. I

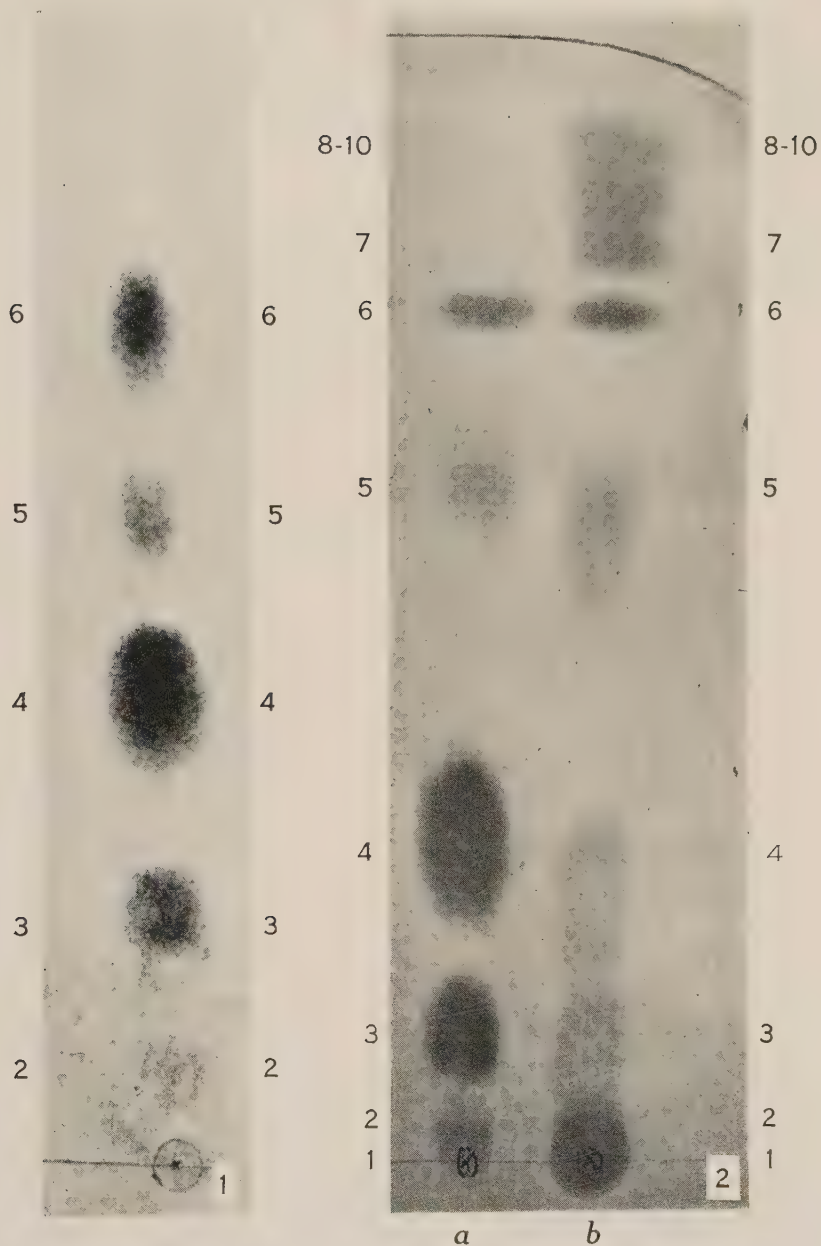


Fig. 1.—Paper chromatogram run with caprylic alcohol-oxalic acid, showing ferric hydroxamate spots derived from esterified acids from apples (1949).

Fig. 2.—Paper chromatogram run with benzene-acetic acid showing ferric hydroxamate spots derived from:

(a) Esterified acids from apples (1949);

(b) An artificial mixture of esters containing acids with one to ten carbon atoms.

The apple volatile products were collected as two samples, the less volatile (1) in an ice-calcium chloride trap, and the more volatile (2) in a liquid-air trap.

The mass spectra of these samples were measured, when it was found that the first was heavily contaminated with water, and the second with CO_2 . It did not prove possible to separate the water by fractionation, but the CO_2 could be removed by this method.

The mass spectrum of the more volatile sample (2) after fractionation was complex, prominent peaks being observed at masses 74, 102, 116, 130, 136, 206. It was thought that the samples contained aliphatic esters, and examination of the spectra of a number of such esters with parent peaks at these values indicated that ethyl *n*- and *isobutyrate*, and ethyl *n*- and *isovalerate* were more probable than esters such as *n*-butyl acetate. This evidence was, however, not conclusive.

TABLE 3
COMPARISON OF MASS SPECTRA OF SAMPLES A AND B WITH THOSE OF
ALIPHATIC MONOCARBOXYLIC ACIDS

Mass	Ion Peak Heights					Sum of Mass Spectra of Pure Acids
	Sample	Acetic	Propionic	<i>n</i> -Butyric	<i>n</i> -Valeric	
Sample A						
43	249	49	110	265	27	451
45	357	45	180	133	14	372
60	466	29		375	47	451
73	205		120	106	16	242
74	205		198	6	4	208
87	20			6	1	7
88	17			16		16
Sample B						
43	674	692	37			729
45	588	625	61			686
60	422	422				422
73	43		40			40
74	66		66			66

At this stage the volatiles of type RCOOR' were hydrolysed to the corresponding alcohols $\text{R}'\text{OH}$ and acids RCOOH , which were separated by distillation. The acids were converted to the sodium salts, sample A, and the alcohols oxidized with chromic acid to the corresponding acids, these also being converted to the sodium salts, sample B.

The free acids were obtained when desired for analysis by treatment of the Na salts in vacuo with vacuum-dried H_3PO_4 , and the mass spectra of the acids in samples A and B measured.

The mass spectra of purified samples of the aliphatic acids acetic, propionic, *n*- and *isobutyric* and *n*- and *isovaleric* were obtained and compared with the spectra of samples A and B. (Table 3.) The *iso*- acids gave large peaks at mass numbers where there were none observed in the spectra of the acids from samples A and B, so it was assumed that only normal acids were present in these.

The agreement obtained between the sum of the patterns of the aliphatic acids and the patterns produced by the samples is not very good; this is probably due to the fact that the acids employed for obtaining the calibration spectra were not of high purity. Nevertheless, the results indicate that sample A probably contains acetic, propionic, *n*-butyric, and *n*-valeric acids, and that sample B contains acetic and propionic acids as major constituents.

The esters present in the original sample would therefore be ethyl and propyl acetate, propionate, *n*-butyrate, and *n*-valerate.

HYDROGEN PEROXIDE IN THE ENZYMIC OXIDATION OF HETEROAUXIN

By P. L. GOLDACRE*

[*Manuscript received March 15, 1951*]

Summary

A hydrogen peroxide-peroxidase system is essential to the enzymic oxidation of indole-3-acetic acid. Catalase and colloidal platinum inhibited strongly such oxidation, and extremely low concentrations of guaiacol competed with the I.A.A. for the I.A.A. oxidase.

Added low concentrations of hydrogen peroxide enhanced the enzymic oxidation of I.A.A. The endogenous hydrogen peroxide does not arise from external flavoprotein oxidations; it probably does not arise as a co-product in the oxidation of I.A.A. itself or of an oxidation product of I.A.A.

2,4-D opposed the effect of added catalase and inhibited pea epicotyl catalase approximately 30 per cent. at $10^{-3}M$ concentration. It had no influence on the peroxidase.

Boiled onion bulb juice strongly inhibited the peroxidase. Boiled pea epicotyl juice did not.

I. INTRODUCTION

An enzyme system from etiolated plant tissues capable of oxidizing and inactivating indole-3-acetic acid (heteroauxin, I.A.A.) has been described by Tang and Bonner (1947, 1948) and studied by Wagenknecht and Burris (1950). In an earlier communication (Goldacre 1949) it was shown that the activity of this system was enhanced by the presence of the synthetic plant growth regulator, 2,4-dichlorophenoxyacetic acid (2,4-D). It was of interest to examine the manner in which this stimulation arose. The possibility of 2,4-D behaving as an artificial redox carrier was considered, but the molecule is rather stable to oxidation and reduction, and no polarographic step could be obtained between applied voltages of +2.00 and -2.00 (*versus* the saturated calomel electrode) under a wide range of pH and with a variety of supporting electrolytes. This has also been the experience of Velstra (1944) using several other plant growth regulators.

The enzyme system is strongly inhibited by a heat-stable substance present in pea epicotyl tissue (Tang and Bonner 1948) and 2,4-D opposes this inhibition. It was suggested (Goldacre 1949) that 2,4-D may act by counteracting the effect of the natural inhibitor. However, it was subsequently found that after removing the inhibitor by precipitation of the enzyme with acetone, 2,4-D still increased the activity of the system. A study was therefore made into the nature of the I.A.A. oxidase system. This paper reports some of the information obtained.

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II. EXPERIMENTAL

(a) Enzyme Preparations

Seeds of green peas (variety William Massey) were stored dusted with "Spergon" (tetrahydroxybenzoquinone) to control the growth of moulds. Seeds were soaked in tap water for two hours, sown in sand in flats and incubated at 24°C. in complete darkness. After seven to ten days, when the epicotyls were 10-15 cm. long, they were harvested and ground for three minutes in approximately half their weight of ice-cold distilled water in an ice-jacketed metal Waring Blender. The juice was squeezed through muslin and filtered in the cold. The filtrate was used as "crude I.A.A. oxidase" in many experiments.

To concentrate the enzyme system and free it from an inhibitor present in the tissue (Tang and Bonner 1948) 0.44 volumes of ice-cold acetone were added to the cold filtrate. The precipitate was centrifuged down, washed twice with cold 40 per cent. acetone, and shaken in a small volume of M/15 phosphate buffer, pH 6.64. This was centrifuged and the supernatant used as "acetone-precipitated" enzyme. Most experiments reported here have been carried out both with crude and acetone-precipitated enzyme.

(b) Activity Measurement

The activity of the I.A.A. oxidase was measured by determining the residual I.A.A. concentration after 20 minutes' incubation at 27.5°C. During this time progress curves were essentially linear. I.A.A. was determined by means of the $\text{FeCl}_3\text{-H}_2\text{SO}_4$ reagent of Tang and Bonner (1947). The intense cherry-red colour rises to a maximum optical density and then slowly fades. Not only the rate of development and fading but also the maximum intensity reached depend upon the room temperature (Goldacre, unpublished data). Readings were therefore made at a time after adding the reagent when, according to the room temperature, the colour would be at its maximum intensity. A series of standards was included in every run.

(c) Oxidation of I.A.A.

According to Tang and Bonner (1947) each mole of I.A.A. consumes one mole of oxygen and produces one mole of carbon dioxide, the indole nucleus remaining intact. The overall equation fitting these observations is shown in Figure 1.

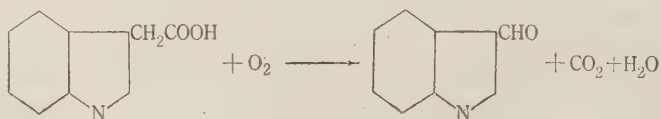
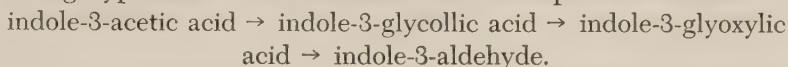


Fig. 1

It is possible to prepare the 2,4-dinitrophenylhydrazone of a neutral, ether-extractable substance which increases in concentration as the reaction proceeds and which is presumed to be indole-3-aldehyde. It is usual for biological

oxidations to proceed by two hydrogen equivalents per step so it is probable that two oxidations and one decarboxylation are involved in this conversion. As a working hypothesis it was assumed that the steps involved are:



Felber (1948) demonstrated cytologically that protruberances induced on bean tissue by treatment with 2,4-D had greater peroxidase activity than untreated tissue. It has also been shown (Goldacre 1949) that 2,4-D increases the *in vitro* activity of I.A.A. oxidase. Further, Tang and Bonner (1947) indicated that the I.A.A. oxidizing system may contain a haem enzyme, and the system is poisoned by peroxidase inhibitors such as cyanide, azide, and hydroxylamine. These facts suggested that one of the oxidation steps in the I.A.A. oxidase system may be a peroxidase. Both crude and acetone-precipitated enzyme preparations do in fact have strong peroxidase activity, and in the presence of 0.05M hydrogen peroxide vigorously oxidized solutions of guaiacol, guaiacum resin, benzidine, pyrogallol, *p*-phenylene diamine, *o*-cresol, *m*-cresol, and *p*-cresol. Pea epicotyl brei gave the red colour of tetraguaiacoquinone on adding guaiacol even in the absence of added hydrogen peroxide.

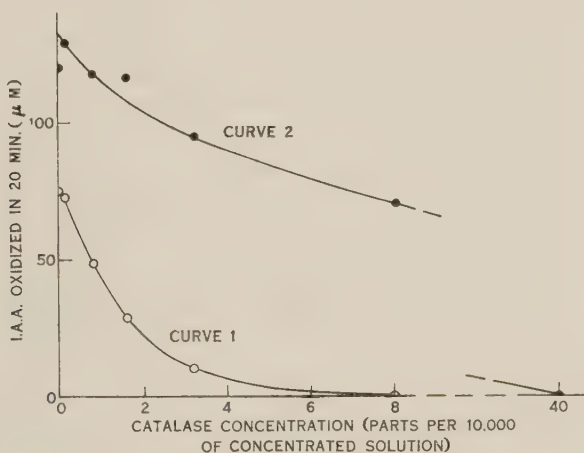


Fig. 2.—Effect of catalase on the I.A.A. oxidase (curve 1) and the influence of 2,4-D on this effect (curve 2). Tubes loaded with 0.5 ml. M/15 phosphate buffer, pH = 6.64; 0.5 ml. crude enzyme; 0.5 ml. 10^{-3} M I.A.A.; catalase; 2×10^{-3} M 2,4-D; and distilled water to a total volume of 2.5 ml. Incubated 20 min. at 27.5°C.

(d) Effect of Hydrogen Peroxide-Consuming Systems

A highly active purified catalase preparation was made from sheeps' livers according to Von Euler and Josephson (1927) and had a Katalasefähigkeit (K.f.) value of approximately 4100. This proved to be highly inhibitory to the I.A.A. oxidase system, even in low concentrations. This is shown in Figure 2, curve 1. 2,4-D reversed the inhibitory effect of catalase (Fig. 2, curve 2).

Boiling the catalase solution for 15 minutes to destroy its peroxide-splitting activity also destroyed its inhibiting effect.

In addition, a suspension of colloidal platinum, high in H_2O_2 -splitting activity, was prepared by arcing under distilled water two stout platinum electrodes using 240 volts A.C. and a 1000 watts series resistor in circuit. This suspension also inhibited the I.A.A. oxidase system strongly (Fig. 3).

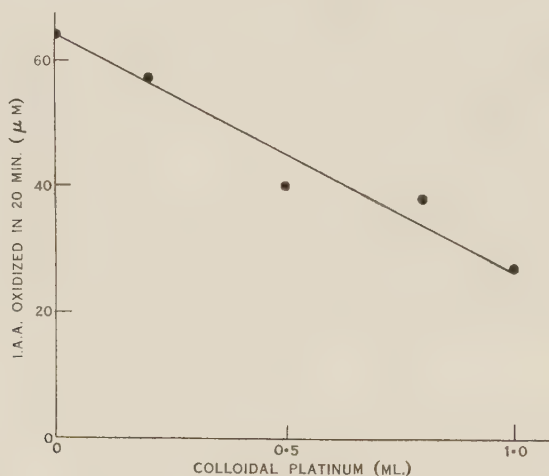


Fig. 3.—Effect of colloidal platinum on I.A.A. oxidase. Colloidal platinum prepared by Bredig's method. Tubes loaded with 0.5 ml. M/15 phosphate buffer, pH = 6.64; 0.5 ml. crude enzyme; 0.5 ml. 10^{-3}M I.A.A.; colloidal platinum as shown; and distilled water to a total volume of 2.5 ml. Incubated 20 min. at 27.5°C . Series of standards contained concentrations of colloidal platinum to take into account photo-absorption due to this substance.

This is strong evidence that hydrogen peroxide, which is specifically and rapidly decomposed by catalase and by colloidal platinum, is essential to the enzymic oxidation of I.A.A. Confirmatory evidence was obtained by observing strong inhibition of I.A.A. oxidation by extremely low concentrations of guaiacol, a substrate specific for peroxidases. The Michaelis-type curve relating activity to substrate (I.A.A.) concentration was converted in the presence of $5 \times 10^{-6}\text{M}$ guaiacol to a sigmoid curve typical of the competitive type of inhibition (Fig. 4). When plotted as reciprocals (Fig. 5) two straight lines were obtained having significantly different slopes, indicating that the $K_s/(S)V$ term of the equation relating reaction velocity to substrate concentration,

$$\frac{1}{v} = \frac{1}{(S)} \cdot \frac{K_s}{V} + \frac{1}{V},$$

where v = observed initial velocity, (S) = substrate concentration, K_s = Michaelis constant, and V = limiting velocity, has been increased and hence that the inhibitor is competing with the substrate.

We may therefore conclude that H_2O_2 -peroxidase system is essential to the enzymic oxidation of I.A.A.

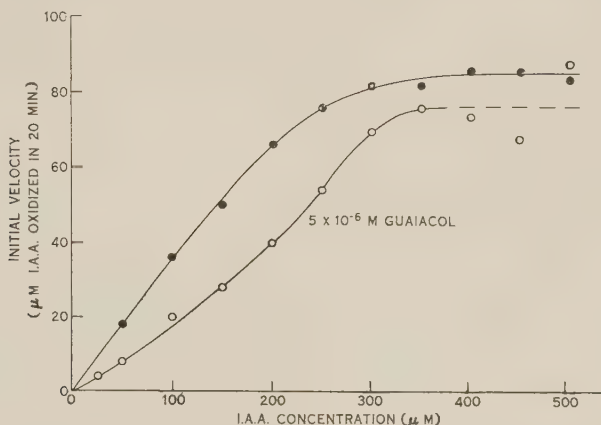


Fig. 4.—Competitive inhibition of I.A.A. oxidase by guaiacol. Tubes loaded with 0.5 ml. M/15 phosphate buffer, pH = 6.64; 0.5 ml. crude enzyme; I.A.A. in concentrations shown; 5×10^{-6} M guaiacol for the curve indicated; and distilled water to a total volume of 2.5 ml. Incubated 20 min. at 27.5°C. Guaiacol in this concentration had no influence on the chemical determination of I.A.A.

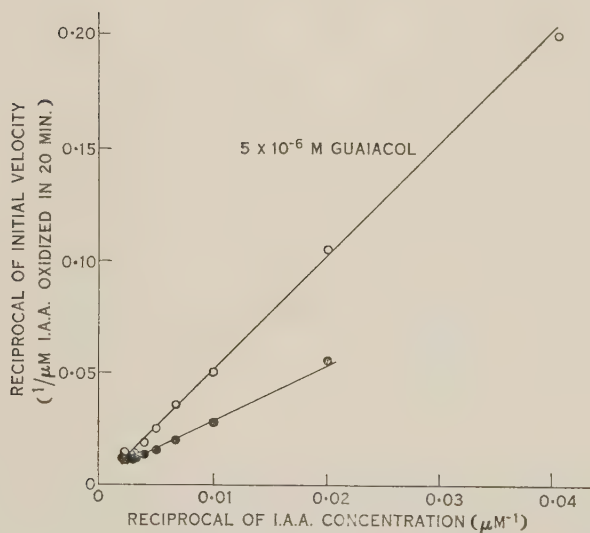


Fig. 5.—The data of Figure 4 plotted as reciprocals.

(e) Effect of Added Hydrogen Peroxide

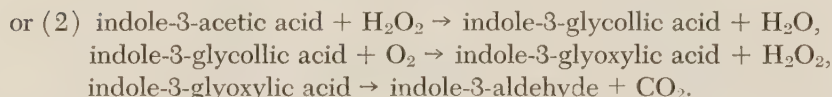
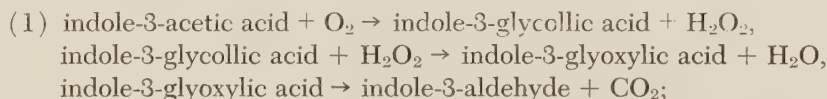
Hydrogen peroxide, like most strong oxidizing agents, rapidly destroys I.A.A. However, at concentrations below 0.02N the non-enzymic oxidation of

I.A.A. during 20 minutes incubation at 27.5°C. is negligible. In the presence of crude pea epicotyl enzyme, the activity was increased 28 per cent. by 0.0014N added H_2O_2 and 61 per cent. by 0.014N added H_2O_2 .

(f) *The Source of Endogenous Hydrogen Peroxide*

Hydrogen peroxide is known to arise from molecular oxygen during oxidation by specific flavoprotein enzymes of substrates such as xanthine, aldehydes, *D*-amino acids, reduced coenzyme 1, uric acid, glucose, etc. The addition of $1.25 \times 10^{-3}\text{M}$ xanthine, $1.25 \times 10^{-3}\text{M}$ hypoxanthine, $1.25 \times 10^{-3}\text{M}$ adenine, 10^{-2}M glucose or $2.5 \times 10^{-3}\text{M}$ *D,L*-alanine to an acetone-precipitated I.A.A. oxidase preparation resulted in no increase in the rate of enzymic oxidation of I.A.A. Benzoate (10^{-2}M), an inhibitor for *D*-amino acid oxidase, showed no inhibition. It appears that no enzyme capable of utilizing these substrates to produce H_2O_2 is present in the preparation, though the preparation has the capacity to oxidize I.A.A. by means of H_2O_2 .

Moreover, one mole of I.A.A. consumes one mole of oxygen and produces one mole of carbon dioxide (Tang and Bonner 1947). Thus if oxygen were first utilized by an external flavoprotein oxidase system to form hydrogen peroxide which in turn oxidized I.A.A., *two* moles of oxygen would be required. The possibility was considered that hydrogen peroxide may be produced in one of the oxidation steps of the I.A.A. oxidase system and subsequently utilized in the other step, e.g.



However, it is known that if I.A.A. is left for long enough in contact with the enzyme it becomes *totally* oxidized. Either of the above schemes would require that hydrogen peroxide formed in one reaction be *quantitatively* available for the second. Owing to the instability of hydrogen peroxide in tissue extracts containing traces of catalase it seems unlikely that this condition should ever be achieved. The origin of the hydrogen peroxide is at present being investigated.

(g) *Effect of 2,4-D on Catalase*

Crude and acetone-precipitated I.A.A. oxidase preparations each contained weak catalase activity. Since hydrogen peroxide is essential to the enzymic oxidation of I.A.A. and since 2,4-D opposes the inhibitory effect of catalase on the system (Fig. 2), it was reasonable to suppose that the accelerating effect due to 2,4-D may be attributed to its inhibition of the catalase present in the I.A.A. oxidase preparations.

This was tested in the following manner. Catalase activity was measured by adding a volume of diluted enzyme to 10 ml. M/15 phosphate buffer, pH

6.8, 25 ml. approximately 0.1N H_2O_2 , addenda, and water to a total volume of 41 ml. in an ice bath at 0°C . At time intervals 4 ml. aliquots were pipetted into 2 ml. 2N H_2SO_4 and titrated with 0.05N KMnO_4 . Typical logarithmic decay curves were obtained. Monomolecular velocity constants were determined for each time interval using the relationship

$$k = \frac{1}{t} \log_{10} \frac{a}{a-x}$$

where a = original concentration of hydrogen peroxide, x = concentration of H_2O_2 decomposed in t minutes, and extrapolated to zero time. This value was used as a measure of the catalase activity.

Using first the catalase concentrate prepared from sheeps' livers, the concentration range in which activity is proportional to enzyme concentration was first established. This is a prerequisite to testing for non-competitive inhibition. Within this range, concentrations of 2,4-D up to $1.25 \times 10^{-3}\text{M}$ produced a maximum of 30 per cent. inhibition.

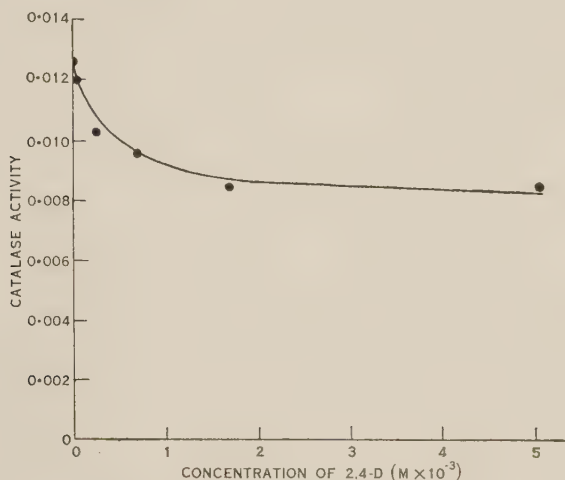


Fig. 6.—Effect of 2,4-D on the catalase activity of the acetone-precipitated I.A.A. oxidase preparation from etiolated pea epicotyls. Catalase activity is given by $\log a/(a-x)$ per min. at zero time, where a = original conc. of H_2O_2 , x = conc. of H_2O_2 decomposed in t minutes.

Repeated with an acetone-precipitated I.A.A. oxidase preparation showing weak catalase activity, very similar results were obtained. Figure 6 shows up to 32 per cent. inhibition for concentrations of $2 \times 10^{-3}\text{M}$ 2,4-D and higher. Increasing the concentration results in no greater inhibition. When catalase activity was measured manometrically under the same conditions of temperature and pH (27.5°C ., pH 6.64) as those under which the stimulation in the rate of I.A.A. oxidation was observed, essentially similar results were obtained.

From this information it cannot be predicted with certainty whether the measured inhibition of catalase by 2,4-D is sufficient to account for the observed increase in the rate of I.A.A. oxidation, or whether 2,4-D may in other ways influence the availability of hydrogen peroxide. Kinetic treatment involves a knowledge of the H_2O_2 concentration during the course of the reaction, and since this work was done, Galston, Bonner, and Baker (1951) indicate that light and possibly other factors may influence the production of H_2O_2 . However, it is clear from Figure 2 that the rate of reduction of I.A.A. oxidase activity with increasing catalase concentration is markedly diminished in the presence of 2,4-D, indicating that this substance in some way makes more H_2O_2 available for the I.A.A. oxidation reaction.

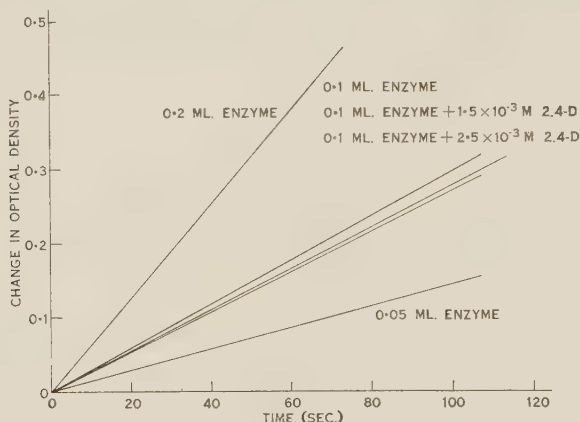


Fig. 7.—Progress curves for activity determination of peroxidase by the method of Ponting and Joslyn (1948). Temperature 19°C . Crude blended pea epicotyls diluted 1 in 10. Spectrophotometer cuvettes loaded with 5 ml. 0.2M acetate buffer, pH 5.0; 1 ml. approximately 0.1N H_2O_2 ; 1 ml. 0.04M guaiacol; the volume of diluted enzyme shown; and water to a total volume of 20 ml. Time rate of increase in optical density measured at 425 $\text{m}\mu$.

(h) Effect of 2,4-D on Peroxidase

Since I.A.A. is oxidized by a non-specific peroxidase capable of oxidizing guaiacol in the presence of H_2O_2 , a convenient way of studying separately the peroxidase fraction of the I.A.A. oxidase system proved to be the method of Ponting and Joslyn (1948), which depends on measuring the time rate of increase in optical density at a wavelength of 425 $\text{m}\mu$, using 0.002M guaiacol and 0.05N H_2O_2 as substrates for the enzyme. Guaiacol becomes oxidized to the red tetraguaiacoquinone. Progress curves were precisely linear for long periods and the slopes of these curves ($\log I_0/I_t$ per sec.) were used as a measure of the reaction velocities.

This method was used to examine whether 2,4-D had any direct stimulatory effect on the peroxidase itself. Using diluted whole pea epicotyl cytoplasm, the range of rate-limiting peroxidase concentrations was determined (Fig. 7), and within this range, concentrations of 2,4-D up to $2.5 \times 10^{-3}\text{M}$ had no effect.

However, it should be remembered that in these experiments H_2O_2 is present in excess while the concentration during the *in vitro* oxidation of I.A.A. is very low and possibly at times rate-limiting. Existing methods do not permit the measurement of catalase and peroxidase activity under these conditions.

(i) *Effect of Etiolated Pea Epicotyl Inhibitor and of Onion Bulb Inhibitor on Peroxidase*

It is of interest to determine whether the strong inhibitions produced by substances present in plant tissues (Tang and Bonner 1948; Goldacre 1949) occur at the peroxidase step. Boiled inhibitor solutions were prepared by blending the tissue in a minimum volume of distilled water, boiling for 15 minutes, and filtering. Peroxidase activity was measured by the method of Ponting and Joslyn (1948), working in the rate-limiting range of enzyme concentration.

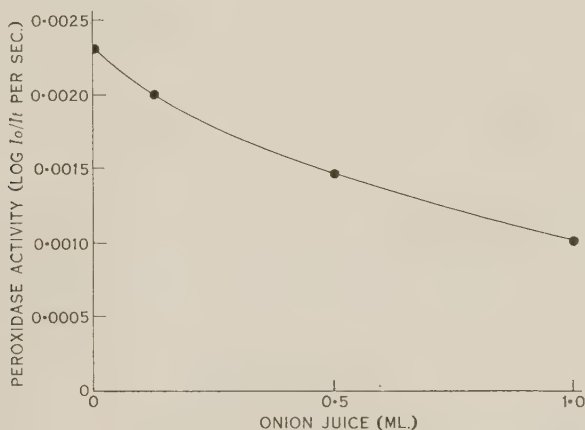


Fig. 8.—Effect of boiled onion juice on the peroxidase. Activity measured by method of Ponting and Joslyn (1948). Crude blended pea epicotyls diluted 1 in 10 (0.1 ml.) were used in a total volume of 20 ml. Temperature 19°C .

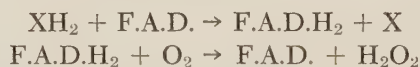
With pea inhibitor, no influence on the activity was obtained with concentration up to 100 times that associated with the enzyme in the tissue. Onion juice, however, produced strong inhibitions, shown in Figure 8.

Thus the inhibitory nature of these two plant extracts is not the same. Pea extracts may be concerned with the hydrogen peroxide-producing reaction while onion juice inhibits the peroxidase.

III. DISCUSSION

It has been shown that hydrogen peroxide-consuming systems strongly inhibit the enzymic oxidation of I.A.A. Catalase and colloidal platinum, which catalyse the dismutation of hydrogen peroxide, depress the rate of oxidation of I.A.A., presumably by reducing the availability of peroxide for this reaction. Guaiacol, a substrate requiring hydrogen peroxide plus peroxidase for its oxidation, produces marked inhibition in a competitive fashion of I.A.A. oxidation. Thus it is concluded that a hydrogen peroxide-peroxidase system is essential to the enzymic oxidation of I.A.A. Added low concentrations of H_2O_2 enhance the rate of oxidation.

The source of the hydrogen peroxide is not known, but reasons have been advanced to show that it does not arise from an external flavoprotein oxidation:



and probably not from the intermediary oxidation of I.A.A. or a product from it. Galston, Bonner, and Baker (1951) present evidence suggesting that peroxide formation is a function of light, and this aspect is now under examination.

2,4-D markedly diminishes the effect of catalase, particularly at relatively higher catalase concentrations (Fig. 2), in some way making available more peroxide for the I.A.A. oxidation reaction. 2,4-D inhibits both liver catalase and pea epicotyl catalase to the extent of about 30 per cent., but it is not yet known whether this is sufficient to account for the effect of 2,4-D on the oxidation of I.A.A. This must await further information on the source and concentration of peroxide in the reaction.

The oxidation products of I.A.A. are not yet known. It seems probable that the end product is indole-3-aldehyde but the intermediates have not yet been isolated or identified; as a working hypothesis indole-3-glycollic and indole-3-glyoxylic acids have been suggested. Nothing is yet known of the decarboxylation reaction.

IV. REFERENCES

- FELBER, I. M. (1948).—*Amer. J. Bot.* **35**: 557.
 GALSTON, A. W., BONNER, J., and BAKER, R. S. (1951).—*Arch. Biochem.* (in press).
 GOLDACRE, P. L. (1949).—*Aust. J. Sci. Res. B* **2**: 154.
 PONTING, J. D., and JOSLYN, M. A. (1948).—*Arch. Biochem.* **19**: 47.
 TANG, Y. W., and BONNER, J. (1947).—*Arch. Biochem.* **13**: 11.
 TANG, Y. W., and BONNER, J. (1948).—*Amer. J. Bot.* **35**: 570.
 VELSTRA, H. (1944).—*Enzymologia* **11**: 97, 137.
 VON EULER, H., and JOSEPHSON, K. (1927).—*Liebigs Ann.* **452**: 174.
 WAGENKNECHT, A. C., and BURRIS, R. H. (1950).—*Arch. Biochem.* **25**: 30.

THE EARLY STAGES OF THE OXIDATION OF ADRENALINE IN DILUTE SOLUTION

By E. M. TRAUTNER* and T. R. BRADLEY*

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Summary

The auto-oxidation of adrenaline in concentrations of M/500 to M/10,000 and at pH 6-8 starts after a time lag that is dependent on the conditions but independent of the adrenaline concentration. This is followed by a short period of rapid acceleration of the oxidation rate and then by a period of steady oxidation of constant rate for several hours, proportional to the adrenaline concentration. The solutions are red, later orange to brown, melanin is formed, and the oxygen uptakes, six to seven atoms for one molecule of adrenaline, indicate destructive oxidation beyond opening of the ring structure.

It is suggested that during the time lag the oxidation of adrenaline is started by traces of metallic ions (Fe and Cu) present, adrenochrome being formed in the process and further catalysing the reaction. The acceleration of the oxidation rate and the onset of destructive oxidation are due to the formation of more readily oxidizable compounds of indoxyl structure, the reaction probably involving a free radical mechanism. The later, constant oxidation proceeds over the quinonoid redox systems of adrenochrome and oxo-adrenochrome that are established during the period of acceleration. At this stage the reaction mixture consists of unreacted adrenaline, the quinonoid systems, and of further oxidation products, which act as hydrogen donors towards the quinones. The regulation of the oxidation rate is ascribed to the effect of the hydrogen transfers on the redox potential of the quinonoid systems and possibly to the inhibiting influence of later oxidation products. Finally the ring structures are consumed and, apart from condensation products like melanin, the reaction mixture consists of late oxidation products which without the action of redox systems, undergo further atmospheric oxidation. Tentative formulations of the earlier steps of adrenaline oxidation are proposed.

The influence of catalysts, heavy metal ions (Fe and Cu, also haematin and methaemoglobin), and adrenochrome is confined to the initial stages of the reaction sequence; they shorten the time lag and cause a transient period of more rapid oxygen uptake, the duration of which depends on their concentration. In catalytic amounts they do not appreciably affect the rate of the final oxidation.

The auto-oxidation of adrenochrome begins after a time lag and shows a period of more rapid oxidation before the final steady oxidation rate is established.

Oxidizing red adrenaline solutions or adrenochrome solutions form strongly fluorescent colourless to yellow solutions on reduction and on addition of free alkali with or without the admission of air. It is suggested that, depending on the conditions, leuco-adrenochrome or *N*-methyl-5 : 6-dihydroxy-indoxyl, but usually mixtures of both compounds are formed. If at lower pH (7-8.5) adrenaline is allowed to oxidize in the presence of reducing agents (sulphite,

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amino acids, etc.) almost colourless fluorescent solutions are formed; adrenochrome is quickly changed by serum into a green fluorescent solution. The question whether the reaction mixture contains the red quinones or the fluorescent leuco compounds depends on the proportion of primary oxidation to dehydrogenation.

Most polyphenolases oxidize adrenaline beyond the adrenochrome stage. Several preparations from the solanaceous plant *Duboisia myoporoides* were found to cause an uptake of two atoms of oxygen only.

The presence of a peroxidative step in the reaction sequence of adrenaline oxidation and the effect of catalase are mentioned.

In the presence of free alkali, adrenaline rapidly consumes six, adrenochrome four atoms of oxygen per molecule, clear yellow, strongly fluorescent solutions being formed. This period of rapid oxygen uptake is followed by a prolonged period of slow uptake, two to three more atoms of oxygen being consumed. During this period the solutions are clear yellow but without any fluorescence. Neither red quinones nor melanin are formed under these conditions.

Age, colour, fluorescence, or oxygen uptakes of an oxidizing adrenaline solution can give no reliable indication as to its composition. If the physiological action of an adrenaline oxidation product is to be investigated, synthetic intermediates will have to be chosen. Tentative methods for the preparation of comparatively pure solutions of the fluorescent compounds are proposed.

I. INTRODUCTION

In recent years several publications have described physiological effects of somewhat indefinite oxidation products of adrenaline, or merely of "aged solutions," "oxidized adrenaline," or "coloured adrenaline" (cf. review by Bacq 1949). The observations often appear contradictory and are not always reproducible; their interpretation presents difficulties since the composition of the solutions has not been ascertained.

The first steps of adrenaline oxidation are usually presented as proceeding from adrenaline to adrenaline-quinone, leuco-adrenochrome, and adrenochrome (Fig. 22, I-IV†). Doubts concerning this sequence have been expressed by West (1947), Bacq (1949), and others, and will be discussed later. A formulation of the mechanism of the oxidation beyond adrenochrome has not yet been proposed. The oxidation of adrenaline has been investigated with the purpose of establishing to what degree colour, fluorescence, oxygen uptakes, or age of an oxidizing solution give an indication of its composition. The mechanism of adrenaline oxidation at physiological pH is presented as proceeding first to *o*-quinones and the corresponding diphenols. It is suggested that the destructive oxidation mechanism starts through disproportionation of free radicals and proceeds further by hydrogen transfer from later oxidation products to the quinones.

† I, Adrenaline; II, adrenaline-quinone; III, leuco-adrenochrome; IV, adrenochrome; IV_a, quinone-immonium structure of adrenochrome in acid solution; IV*, semiquinone of the adrenochrome system; IV**₂, hypothetical di-radical; V, *N*-methyl-5 : 6-dihydroxy-indoxyl; VI, oxo-adrenochrome; VII, *N*-methyl-5 : 6-dihydroxy-indole.

II. MATERIALS AND METHODS

Crystalline adrenaline was supplied by H. Francis and Co., Melbourne. The preparations showed the correct melting point (211-212°C.) and the experimental results were not modified by further purification.

Adrenochrome was prepared by the method of Randall (1946).

Polyphenolase from the fresh leaves of the solanaceous plant *Duboisia myoporoides* was prepared as described by Trautner and Roberts (1950).

Catalase was prepared from rat liver homogenized with 10 volumes of M/100 phosphate buffer, pH 7.0, and centrifuged. One ml. of a 1 : 50 dilution of the supernatant was used. In some experiments crystalline beef liver catalase prepared by the method of Sumner and Dounce (1939) was reacted in parallel experiments.

Oxygen uptakes were measured in Warburg manometers under the conditions described. Final fluid volume was 5 ml. in all experiments. To allow of better comparison of the results the graphs are, as far as practicable, scaled for oxygen uptakes corresponding to 1 ml. M/100 solution of the substrate to be oxidized (adrenaline, adrenochrome, etc.).

TABLE 1

VISIBLE CHANGES ON SLOW ATMOSPHERIC OXIDATION OF M/500 ADRENALINE IN M/100 BUFFER OR SALT SOLUTION IN TEST TUBES WITH OCCASIONAL AGITATION ON STANDING AT ROOM TEMPERATURE (23°C.)

Time	H ₃ PO ₄	NaH ₂ PO ₄	Buffer pH				
			8.0	Na ₂ HPO ₄	NaHCO ₃	Na ₂ CO ₃	NaOH
On mixing	None	None	None	None	Pink	Pink	Pink
5 min.	None	None	None	Pink	Pink	Yellow	Yellow
1 hr.	None	None	Pink	Pink	Pink	Yellow	Yellow
3 hr.	None	None	Orange	Orange	Yellow	Yellow	Yellow
5 hr.	None	None	Red	Red-brown	Yellow	Yellow	Yellow
20 hr.	None	None	Dark brown	Brown	Yellow	Yellow	Yellow

Volatile amines evolved during oxidation of adrenaline or adrenochrome were determined by the diffusion method of Conway (1947), using boric acid mixture in the centre well.

Colour density was measured with a Lumetron colorimeter (Photovolt, New York), using a green filter for adrenochrome according to the method of Evans and Raper (1937).

Fluorescence was measured with a Klett fluorimeter against eosin solutions as standard, as suggested by West (1947).

All reagents used were of reagent quality. Fresh solutions were prepared for each experiment and the experiments were put on as soon as the flasks were ready. Since the oxidation of adrenaline is strongly catalysed by heavy metal ions, the vessels were washed with glass-distilled water and alcohol and finally steamed. Care was taken to avoid contact of the substances or solutions with metal surfaces, spatulas, etc. Under these conditions reproducible results were obtained.

III. EXPERIMENTAL RESULTS

(a) Oxidation of Adrenaline

(i) Non-enzymic oxidation

Table 1 presents the visible changes observed on slow atmospheric oxidation of M/100 solutions of adrenaline under different conditions at room temperature and with occasional agitation. Higher temperature, stronger aeration, and the presence of catalysts accelerate the development of the changes.

Figure 1 shows the oxygen uptakes of M/2500 adrenaline at different pH levels.

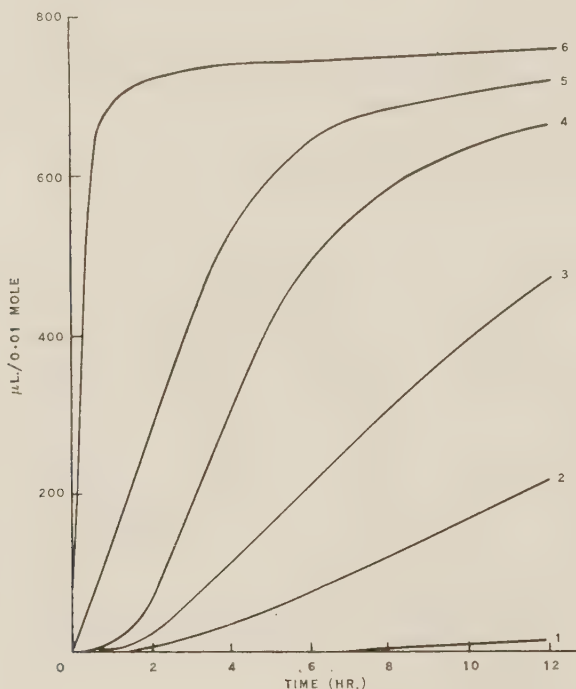


Fig. 1.—Auto-oxidation of adrenaline at various pH.

Final adrenaline concentration M/2500.

Curve 1: 0.1N phosphoric acid at 30°C.

Curve 2: M/15 phosphate buffer, pH 7.0; 23°C.

Curve 3: M/15 phosphate buffer, pH 7.0; 30°C.

Curve 4: M/15 phosphate buffer, pH 8.0; 30°C.

Curve 5: M/15 barbiturate buffer, pH 9.0; 30°C.

Curve 6: 1N caustic soda; 30°C.

Oxidation at alkaline pH.—(1) On oxidation of dilute solutions at pH values over 11 the red colour of adrenochrome is not observed. The solutions turn yellow on admixture of the alkali and show a transient fluorescence, which will be discussed separately. With higher concentrations of adrenaline, turbid brown

solutions are finally obtained, but at the dilutions used the solutions remain clear and neither turbidity nor precipitates are formed. The final pale canary-coloured solutions appear to remain unchanged for days. They do not seem to have any physiological action (Shaw 1941).

The oxygen uptakes show an initial high oxidation rate leading rapidly to the uptake of six atoms of oxygen for one molecule of adrenaline; this period of rapid oxidation is followed by a prolonged period of slow oxidation. No changes in the oxidation rate could be observed at oxidation levels corresponding to the formation of leuco-adrenochrome (two atoms of oxygen); *the alkaline oxidation of adrenaline up to an uptake of six atoms of oxygen proceeds as a steady, continuous function and not in distinct successive steps*. The period of slow oxidation may extend over as much as 24 hours. Owing to the prolonged time of this further reaction, exact measurements of the oxygen uptakes are not possible, but at least two and possibly over three further atoms of oxygen are slowly consumed. No visible changes can be observed during this later period; the solutions remain clear canary yellow.

(2) The reaction mixtures were submitted only to qualitative tests with the purpose of establishing whether the final yellow solutions obtained by different procedures can be regarded as identical. The early fluorescent solutions give positive tests for phenols (ferric chloride, coupling with diazo compounds) and for indole or indoxyl grouping (Ninberg's ninhydrin test, Ehrlich's dimethylaminobenzaldehyde test, the formalin-sulphuric acid test). With the uptake of about six atoms of oxygen for one molecule of adrenaline, the last traces of fluorescence disappear and these tests become negative. Later, ferric chloride gives temporarily a wine-red colour similar to the colour in Gerhard's test for aceto-acetic acid; at the same time a weak Rothera test with nitroprusside is shown, both suggesting the temporary presence of β -keto acids (β -keto-adipic acid has been isolated as a product of bacterial oxidation of aromatic compounds by Kilby 1949). Still later, these tests also become negative and, apart from the change of the yellow colour to a faint pink on acidification, the only simple reaction found was the formation of a slight turbidity with calcium chloride (catechol in alkaline solution is oxidized by permanganate to oxalic acid amongst other products). Part of the nitrogen is finally present in the form of volatile amines; diffusion in Conway units showed up to 50 per cent. adrenaline nitrogen diffusing into the centre well within 24 hours at room temperature on alkaline oxidation.

Oxidation in phosphate buffer at physiological pH.—The solutions obtained are unlike those formed on alkaline oxidation, since under all conditions of oxidation a strong red colour is formed. At intermediate pH, between 10 and 11, this colour is short-lived and soon changes to orange or yellow solutions identical with those of alkaline oxidation. At more neutral pH the colour persists, at first clear and increasing in intensity to a brilliant dark red; after 1-1½ hours, however, it loses its brilliancy and becomes orange-brown to dull brown. Finally, brown solutions or suspensions are formed, which even on prolonged

standing do not appear to undergo any further changes. In Table I the observations indicative of the presence of coloured compounds, orange, red, or brown, are in *italics*; they are confined to pH's between 7 and 8.

(1) Auto-oxidation.—Figure 1, curves 3 to 5, presents the oxygen uptakes of M/2500 adrenaline at pH 7, 8, and 9 at 30°C. The reaction is auto-catalytic and starts after a time lag that amounts to 1-3 hours or more, depending on the conditions. The duration of this lag appears to be, within wide limits, *independ-*

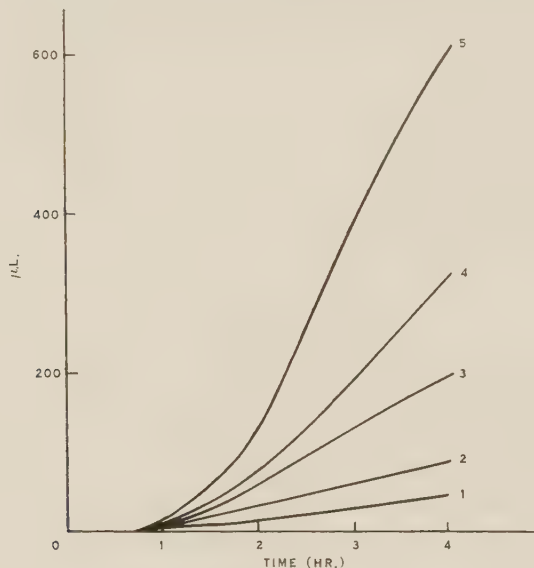


Fig. 2.—Auto-oxidation of adrenaline at various concentrations. M/15 phosphate buffer, pH 8.0; 30°C.
 Curve 1: Adrenaline final concentration M/4000.
 Curve 2: Adrenaline final concentration M/2000.
 Curve 3: Adrenaline final concentration M/1000.
 Curve 4: Adrenaline final concentration M/500.
 Curve 5: Adrenaline final concentration M/250.

dent of the concentration of adrenaline (Fig. 2). Almost abruptly the oxidation rate begins to increase. A constant rate is established after the uptake of about one-half atom of oxygen for one molecule of adrenaline; its magnitude *depends on the concentration of adrenaline* (Fig. 3). The constant rate is maintained for several hours. The oxygen uptakes exceed the adrenochrome level—two atoms for one molecule of adrenaline—and reach four to five atoms before the curves begin to flatten out. The curves do not show the distinct six-atom level observed on alkaline oxidation; rather they appear to exceed it in a smooth, continuous line. The reaction gradually slows down and the total oxygen uptake is between seven and eight atoms, slightly less than that of alkaline oxidation. At higher pH, the curves are S-shaped, showing three distinct sections: the time lag, a period of rapid oxidation, and then the final period of slow oxidation (Fig. 1, curve 4).

(2) Metal catalysis.—The early phases of the oxidation of adrenaline at physiological pH are strongly catalysed by heavy metal ions, particularly ferric and cupric ion. Figure 4 shows the oxygen uptakes recorded under otherwise identical conditions in the presence of different concentrations of metallic ion. The effect of low concentrations of the catalyst consists solely in shortening the time lag; the rate of constant oxidation finally reached remains unaltered. With increasing concentrations of catalyst there appears, *between the time lag and the later period of constant oxidation*, a period of more rapid oxidation, the duration of which increases with the concentration of the metallic ion. The final rate of constant oxidation is almost the same whether metallic ion has been added to the solution or not. Identical series of curves have been obtained by Falk (1949) and Slater (1949) by using various concentrations of haematin or methaemoglobin as catalysts; the latter author noted that the final oxidation rate is not noticeably affected by the amount of catalyst added. It appears also to be independent of the nature of the catalyst as well as of the amounts of oxygen consumed during the intermediate period of rapid oxidation.

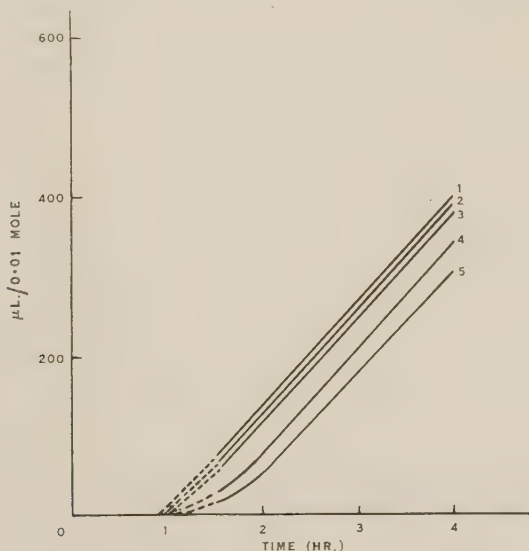


Fig. 3.—Molar oxidation rate. Same experiment as shown in Figure 2; oxygen uptakes plotted for 0.01M adrenaline.

(3) Adrenochrome catalysis.—The addition of adrenochrome, though in 10-20 times the concentration of the metallic ions, causes an identical shortening of the time lag and, possibly, a slight initial acceleration of the oxidation rate (Fig. 5). The final rate of oxidation is not essentially altered. The effect of the catalyst appears to be confined to the first stages of the oxidation. At a proportion of adrenochrome : adrenaline between 1 : 5 and 1 : 10, the constant rate of oxidation is established almost directly after a scarcely noticeable time lag or period of rapid oxidation (Fig. 5, curve 3).

(4) Composition of the solutions.—Apart from the differences in the onset of oxidation and the changes in oxidation rates, the composition of the solutions appears to be the same whether the oxidation has been conducted in the absence or presence of catalysts, and again whether the catalyst was iron, copper, or adrenochrome. Only a few observations are pertinent to the investigation in hand.

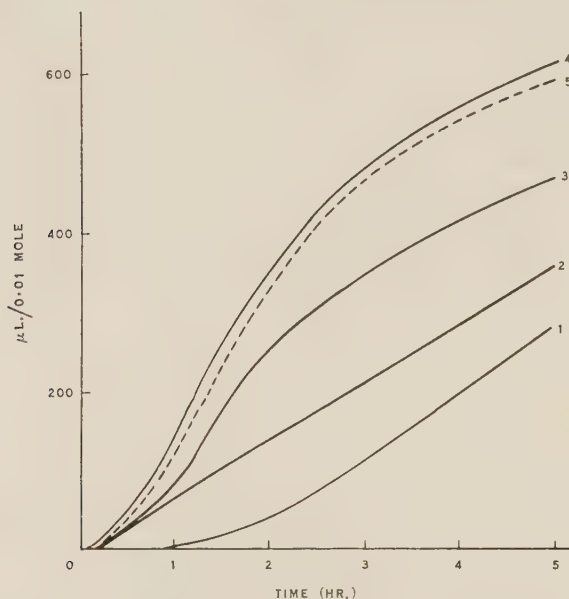


Fig. 4.—Adrenaline oxidation. Metal catalysis. Final adrenaline concentration M/2500; M/15 phosphate buffer, pH 8.0; 37°C.

Curve 1: Adrenaline alone.

Curve 2: Adrenaline with CuSO_4 in final concentration of M/50,000.

Curve 3: Adrenaline with CuSO_4 in final concentration of M/25,000.

Curve 4: Adrenaline with CuSO_4 in final concentration of M/5000.

Curve 5: Adrenaline with FeCl_3 in final concentration of M/5000.

The first visible sign of beginning oxidation consists in the formation of a pink tint; the colour increases quickly to a clear red. These red solutions give quinone reactions and adrenochrome can be separated from the reaction mixture (Green and Richter 1937; Richter and Blaschko 1937). Colorimetric estimation of the adrenochrome present, practicable only so long as the solutions are clear, shows concentrations far below those of an adrenochrome solution equimolecular to the amount of adrenaline initially present. The method—used by Fischer and Lecomte (1949) for the determination of adrenochrome secreted in urine—is not considered very reliable since adrenochrome solutions alone

show slowly increasing colour intensity on standing. The values are probably still too high and indicate only that adrenochrome is not quantitatively formed at an early stage of the oxidation and that brown "melanin" is already formed before the total amount of adrenaline present is oxidized to the adrenochrome stage.

In these turbid brown solutions further oxidation proceeds to an oxygen uptake of seven to eight atoms of oxygen for one molecule of adrenaline, i.e. to the destructive oxidation beyond ring opening of at least a major fraction of the total adrenaline present. The pH of an unbuffered, oxidizing adrenaline solution begins to drop after a few hours and reaches values between pH 4 and 5, indicating the formation of acidic oxidation products (Fig. 6A).

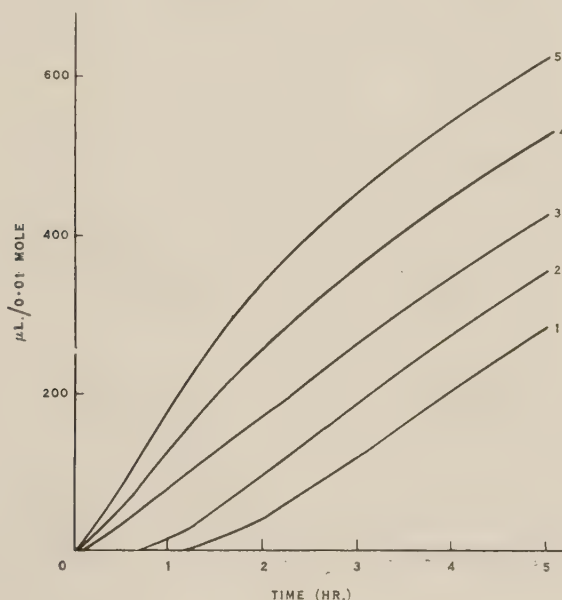


Fig. 5.—Adrenaline oxidation. Adrenochrome catalysis. Final adrenaline concentration M/2500; M/15 phosphate buffer, pH 7.7; 37°C.

Curve 1: Adrenaline alone.

Curve 2: Adrenaline with 10 per cent. adrenochrome.

Curve 3: Adrenaline with 20 per cent. adrenochrome.

Curve 4: Adrenaline with 40 per cent. adrenochrome.

Curve 5: Adrenaline with 80 per cent. adrenochrome.

Hydrogen peroxide is formed during the oxidation of adrenaline (Schales 1938). Hartung (1946) and Falk (1949) suggested that it is formed in the oxidation of leuco-adrenochrome to adrenochrome; no evidence for this location was found in the present work. Figure 7 shows the effect of the addition of catalase to the oxidizing solutions. The amount of oxygen evolved increases as the reaction proceeds, suggesting that *the peroxide-forming step persists*

throughout the whole period of oxidation and is not limited to a distinct early stage. It is doubtful whether hydrogen peroxide plays an essential role in the reaction sequence of the auto-oxidation of adrenaline since the addition of hydrogen peroxide scarcely affects it and that of catalase merely slightly delays it (Fig. 8). The oxidation of adrenaline in the presence of catalase and catalase-containing body fluids is under separate investigation.

If the early clear red solutions are rendered alkaline, the oxidation rate increases instantaneously and the oxygen uptakes follow the pattern of alkaline oxidation leading to clear yellow final solutions. If, however, the later turbid brown solutions are rendered alkaline, the rate and amount of oxygen taken up are less and the solutions remain dirty brown. Once formed these melanins appear to be fairly resistant to further oxidation.

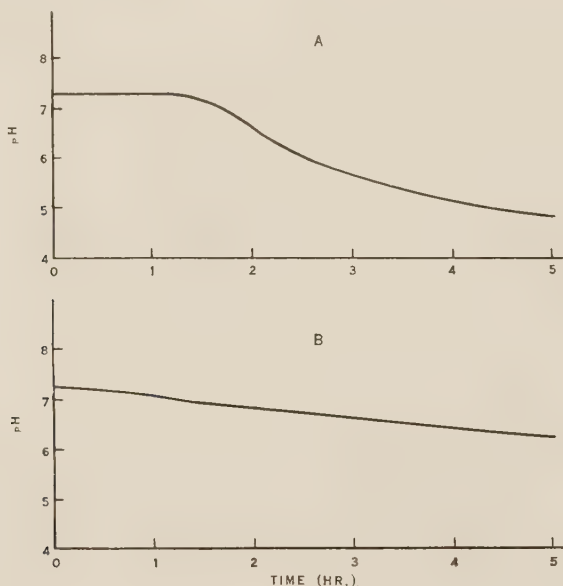


Fig. 6.—A. Changes in pH during adrenaline auto-oxidation. M/100 adrenaline was adjusted to pH 7.2 with a few drops of 0.001N NaOH; 20°C. No buffer was added and oxygen was bubbled through the solution throughout the experiment.

B. Changes in pH during adrenochrome auto-oxidation; same conditions.

Oxidation at acidic pH.—At acidic pH, below 6, the auto-oxidation of adrenaline is very slow and no visible changes or noticeable oxygen uptakes may be observed for a day or more. If a suitable catalyst or oxidizing agent is added, oxidation and colour formation occur. The authors agree, however, with Bacq (1949) that not every red colour observed should be accepted as being due to adrenochrome. For example, the initial red formed at pH 3 by iodate, cited by Bacq, Fischer, and Lecomte (1948) as being inhibited by di-

mercaptopropanol (BAL), soon changes into a stable, deep violet pigment, ioadrenochrome (Richter and Blaschko 1937). The red colour formed at the same pH by persulphate is due to oxo-adrenochrome, synthesized by Cohen (1945). The conditions of the acidic oxidation of adrenaline were not investigated in detail.

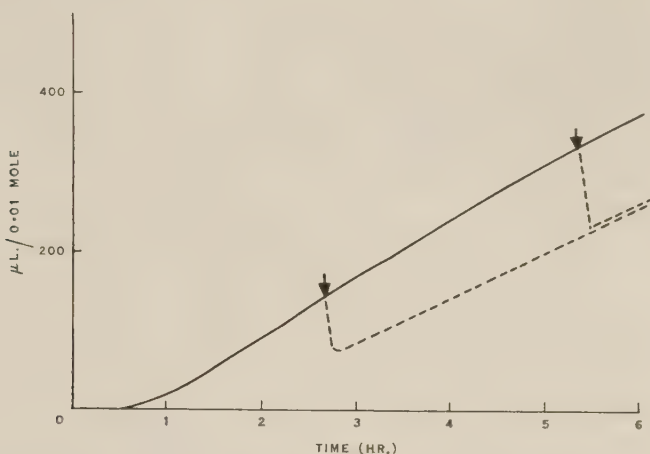


Fig. 7.—Effect of the addition of catalase to adrenaline oxidation mixtures. Final adrenaline concentration M/2500; M/15 phosphate buffer, pH 8.0; 30°C. Catalase added at arrows, 1 ml. of 1 : 50 diluted liver supernatant.

(ii) Enzymic Oxidation

The enzymic oxidation by a polyphenolase at physiological pH is usually quoted as consuming considerably more oxygen than corresponds to the adrenochrome level (cf. James *et al.* 1948). Bacq (1949) says that the auto-oxidation of adrenaline *in vitro* is presumably comparable in its chemical mechanism to its oxidation by catechol oxidase. The present authors obtained, however, acetone powders from the solanaceous plant *Duboisia myoporoides*, which between pH 5.5 and 7.8 and at temperatures between 23° and 30°C. consumed exactly *two atoms of oxygen for one molecule of adrenaline* (Fig. 9, curve 6; Fig. 10, curve 2). The shape of the curves is almost identical with that obtained for the same concentrations of catechol and the final solutions after complete oxidation, 1-1½ hours, are not brown like those obtained by catalytic or auto-oxidation under the same conditions, but clear deep red of a colour intensity equal to, or only slightly less than, that of equimolecular fresh adrenochrome solutions. No difference could be found between them and adrenochrome solutions when in many experiments the enzymic preparation, filtered from the insoluble enzyme, was substituted for adrenochrome solutions of equal concentration.

Not all enzyme preparations, however, showed this theoretical oxidation to adrenochrome; many lead to solutions identical with those obtained on auto-oxidation *except for a higher initial rate of oxygen uptake*. Figure 9 presents the oxygen uptakes of such an "inferior" enzyme at various pH; it is seen that

at pH 5.5 (where the time lag of auto-catalysis may extend to several hours) the oxygen uptake is almost theoretical (Holtz and Kroneberg 1950) but at higher pH a steady rate of further oxidation is maintained *after* the initial period of rapid oxidation. It would appear that enzymic and non-enzymic oxidation are superimposed. No time lag is noticeable in the enzymic oxidation.

Attempts to control the action of an "inferior" enzyme were without success. It was not possible to limit its action to the two-atom level by the addition of catalase, or to arrest the mechanism of further oxidation by the later addition of a good enzyme to the reaction mixture. It would appear that once the mechanism of destructive oxidation has started it follows its course. It was not possible to find any explanation why some enzyme preparations were superior to others.

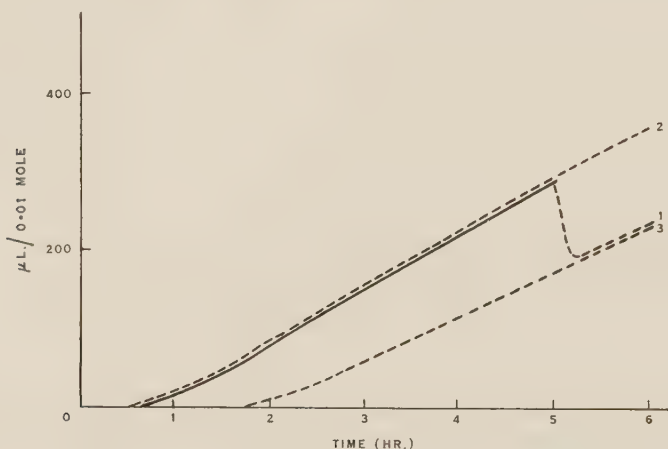


Fig. 8.—Effect of the presence of H_2O_2 or catalase on the course of adrenaline oxidation. Final adrenaline concentration $\text{M}/2500$; $\text{M}/15$ phosphate buffer, pH 8.0; 30°C .

Curve 1: Adrenaline alone; catalase added at end of solid line.

Curve 2: Adrenaline plus 1 ml. $\text{M}/100$ H_2O_2 .

Curve 3: Adrenaline plus catalase, 1 ml. 1 : 50 liver supernatant.

(b) Oxidation of Adrenochrome

(i) Non-Enzymic Oxidation

Table 2 shows the visible changes observed if dilute solutions of adrenochrome ($\text{M}/500$) are allowed to oxidize at room temperature under conditions identical with those shown for adrenaline.

Oxidation at alkaline pH.—Instantaneously on admixture of alkali, adrenochrome loses its red colour; a yellow, strongly fluorescent solution is formed and rapid oxidation sets in. The general shape of the curves of oxygen uptakes is identical with those observed with adrenaline under the same conditions except that two fewer atoms of oxygen are consumed *during the initial period of rapid oxidation* (Fig. 10, curve 1). If adrenaline is first oxidized enzymically to adrenochrome at pH 7.7 and then free alkali added to the reaction mixture, the same oxidation level is reached as if the adrenaline had been directly

oxidized by free alkali (Fig. 10, curves 2 and 3). The rapid initial oxidation is followed by a prolonged, very slow oxygen uptake extending over 24 or more hours, as with adrenaline. The final yellow non-fluorescent solutions were found to be identical, in all tests applied, with those obtained in the alkaline oxidation of adrenaline.

It was investigated whether the formation of the yellow fluorescent compound from adrenochrome on admixture of alkali was due to oxidation. Oxygen was excluded by placing adrenochrome solutions in Thunberg tubes with alkali in the side-arm and evacuating with continuous tapping for as long as 30 minutes before mixing the contents. Under no conditions was it possible to obtain

TABLE 2
VISIBLE CHANGES ON SLOW ATMOSPHERIC OXIDATION OF M/500 ADRENOCROME IN M/100 BUFFER OR SALT SOLUTION WITH OCCASIONAL AGITATION AT ROOM TEMPERATURE (23°C.)

Time	Dil. HCl	Dil. H ₃ PO ₄	NaH ₂ PO ₄	Buffer pH	Na ₂ CO ₃	NaOH
				8.0		
On mixing	Slight fading	Slight fading	No change (red)	No change	No change	Yellow fluores.
10 min.	Dirty green	Dirty green	No change (red)	No change	Greenish fluores.	Yellow
2 hr.	Dirty green	Dirty green	No change (red)	No change	Yellow	Yellow
18 hr.	Dirty green and dark ppt.	Dirty green and dark ppt.	Light green-brown and ppt.	No change	Yellow-brown	Yellow
48 hr.	Dirty green and dark ppt.	Dirty green and dark ppt.	Light green-brown and ppt.	No change	Brown	Yellow
64 hr.	Dirty green and dark ppt.	Dirty green and dark ppt.	Light green-brown and ppt.	Pale brown	Brown	Yellow

a red solution of adrenochrome at alkaline pH; *no oxygen appears to be required for the formation of the fluorescence*. To establish whether the change was reversible, the experiments were repeated in Thunberg tubes with two side-arms, the second containing phosphoric acid equivalent to the amount of alkali in the first so as to bring the alkaline mixture back to neutrality without admission of air. *The change into "fluorescent adrenochrome" is irreversible*; the neutralized solutions are yellow to greenish, strongly fluorescent, and moderately stable. On admission of air they take up oxygen slowly, though faster than adrenaline. The oxygen uptakes on alkalization are identical with those of the original adrenochrome solution, as could be expected. The maximum intensity of fluorescence is of the same order as that observed on alkalization in the presence of air.

Oxidation in phosphate buffer at physiological pH.—The auto-oxidation of adrenochrome proceeds similarly to that of adrenaline but the time lag is shorter and *a temporary early period of rapid oxidation is observed* (Fig. 11).

This latter appears to be characteristic of adrenochrome oxidation. The constant oxidation rate finally reached is slightly lower than that of equimolecular adrenaline solutions. The presence of small amounts of adrenaline exercises a catalytic effect similar to that of small amounts of adrenochrome on adrenaline oxidation: the time lag is shortened; the rate of initial oxidation is possibly slightly increased; but the rate of later oxidation is not essentially altered. In

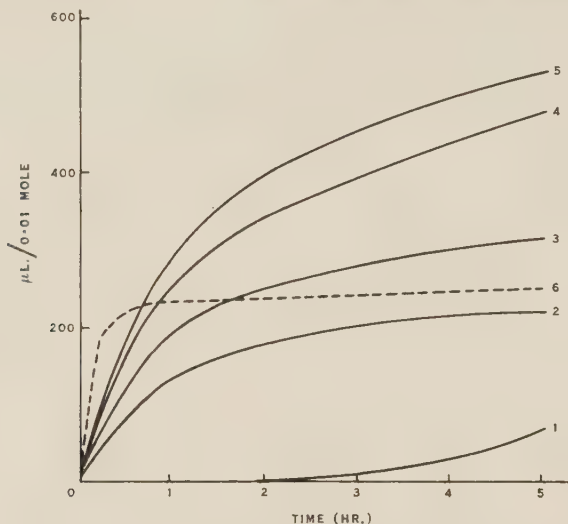


Fig. 9.—Enzymic oxidation of adrenaline. Final adrenaline concentration $M/2500$; $M/15$ phosphate buffer; 23°C . Curves 1-5 show the oxygen uptakes with *Duboisia* polyphenolase, 30 mg., at various pH; curve 6 the uptake with a "purer" enzyme (see text).

Curve 1: Adrenaline alone, pH 7.0.

Curve 2: Adrenaline plus enzyme, pH 5.5.

Curve 3: Adrenaline plus enzyme, pH 6.0.

Curve 4: Adrenaline plus enzyme, pH 7.0.

Curve 5: Adrenaline plus enzyme, pH 8.0.

Curve 6: Adrenaline plus "purer" enzyme, pH 7.0.

proportions of adrenochrome to adrenaline between 1 : 5 and 5 : 1 the same initial catalytic effect is observed but the rate of constant oxidation finally reached is considerably less than the sum of those of the components if singly reacted. The mixtures establish an intermediate oxidation rate (Fig. 12). It would appear that the catalytic effect is confined to the early phases of the reaction.

The pH of an oxidizing, unbuffered adrenochrome solution begins to drop earlier than observed with adrenaline (Fig. 6B).

Oxidation at acidic pH.—Between pH 3 and 4 adrenochrome is within $\frac{1}{2}$ -1 hour converted into a *green compound*, the change soon becoming irreversible; neutralization no longer restores any red colour, but merely a faint pink tint; on alkalization a slight fluorescence is observed, but the oxygen uptakes

are slower and less than those on direct alkalization of adrenochrome. On standing at acidic pH these green solutions deposit within 24 hours a black, unreactive precipitate. Whether this "black melanin" is identical with the "brown melanins" formed at physiological pH was not investigated, nor could it be established whether its formation is accompanied by oxidation. During the first few hours at least *no oxygen is consumed in the formation of "green adrenochrome"* (Fig. 11, curve 1). The instability of red adrenochrome in acid solution makes it still more doubtful whether any red colours observed on acid oxidation of adrenaline are due to adrenochrome. The oxidation of adrenaline to "green adrenochrome" with later formation of black melanin can be effected by acid permanganate.

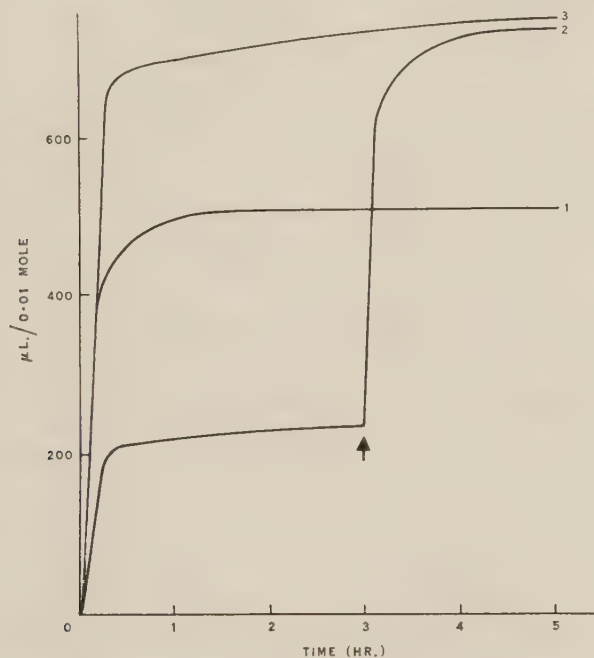


Fig. 10.—Adrenochrome: alkaline oxidation. Final adrenochrome and adrenaline concentrations M/2500; 25°C.

Curve 1: Adrenochrome with 1N NaOH.

Curve 2: Adrenaline first oxidized at pH 7.0 with 30 mg. of a "purer" enzyme, 1 ml. 1N NaOH added at arrow.

Curve 3: Adrenaline with 1N NaOH.

(ii) Enzymic Oxidation

The addition of a polyphenolase does not accelerate the auto-oxidation of pure adrenochrome, and no convincing evidence could be obtained that it accelerates that of aged adrenochrome or of the acidic green or the alkaline yellow solutions if they are brought back to physiological pH. It would appear that the enzyme used reacts rapidly with the catechol grouping of the benzene

ring, but only sluggishly or not at all with those of the nitrogen-substituted adrenochrome system and that it has no effect on the mechanism of the further oxidation of these compounds. If traces of adrenaline are present, an initial rapid oxygen uptake is observed, but then the curves follow their usual course.

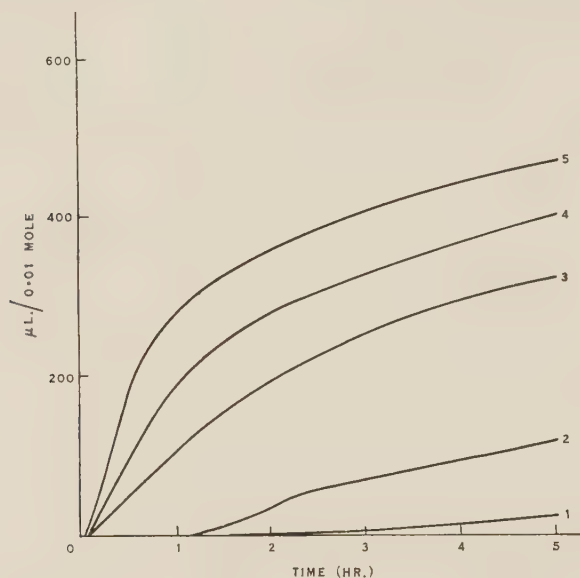


Fig. 11.—Adrenochrome oxidation at various pH. Final adrenochrome concentration M/2500; 37°C.

Curve 1: Adrenochrome in dilute phosphoric acid, pH 4.0.

Curve 2: Adrenochrome in M/15 phosphate buffer, pH 5.0.

Curve 3: Adrenochrome in M/15 phosphate buffer, pH. 6.0.

Curve 4: Adrenochrome in M/15 phosphate buffer, pH 7.0.

Curve 5: Adrenochrome in M/15 phosphate buffer, pH 8.0.

(iii) *Comment on Some Observations on Adrenaline Oxidation*

To shorten the final discussion, three points are separated that are of limited pertinence to the present investigation, namely the enzymic oxidation of adrenaline, the formation of green and fluorescent adrenochrome, and the formation of melanin.

Enzymic oxidation.—The mechanism responsible for the fact that some enzyme preparations stop the oxidation of adrenaline at the two-oxygen stage, with almost quantitative formation of adrenochrome and without any formation of melanin, is outside the scope of this investigation. It is, therefore, only intended to mention the possibilities considered.

(1) Inhibition of metallic catalysts.—Proteins, serum, and other body fluids inhibit the auto-oxidation of adrenaline (Bonhomme 1936). Slater (1949) noticed that small amounts of methaemoglobin inhibit while greater amounts

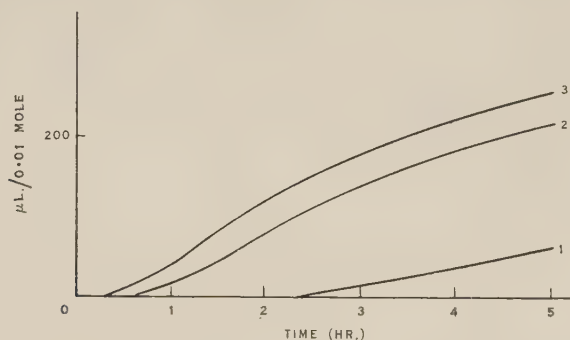


Fig. 12.—Oxidation of an equimolecular mixture of adrenaline and adrenochrome. Final adrenaline and adrenochrome concentrations M/2500; M/15 phosphate buffer, pH 7.0; 25°C.

Curve 1: Adrenaline alone.

Curve 2: Adrenochrome alone.

Curve 3: Adrenaline plus adrenochrome.

catalyse it. It is possible that some component of the acetone powder used combines with and inactivates such traces of metallic ions as are present and necessary to start auto-oxidation. This factor, however, cannot be the only one,

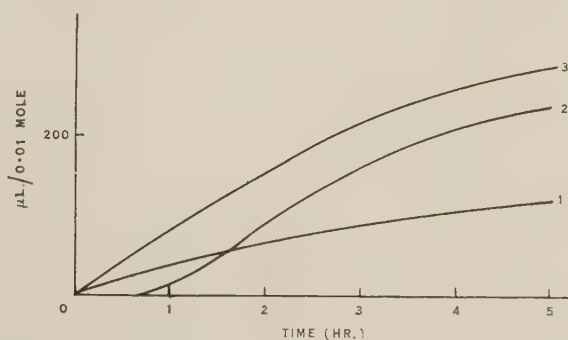


Fig. 13.—Oxidation of an equimolecular mixture of adrenochrome and fluorescent compound. The fluorescent compound was prepared by sodium metabisulphate reduction of adrenochrome, the excess of bisulphite being destroyed by iodine. Final concentration of adrenochrome and fluorescent compound M/2500; M/15 phosphate buffer, pH 7.0; 25°C.

Curve 1: Fluorescent compound alone.

Curve 2: Adrenochrome alone.

Curve 3: Adrenochrome plus fluorescent compound.

since in the action of a good enzyme not only the destructive oxidation caused by metal catalysis but also that caused by adrenochrome catalysis is inhibited.

(2) Lack of hydrogen peroxide.—Nelson and Dawson (1944) in a review on tyrosinase, reach the conclusion that no hydrogen peroxide is formed in the specific action of polyphenolases on phenols and catechols. Since hydrogen peroxide is formed during the auto-oxidation of adrenaline, it may be considered that its presence is essential to start or maintain the mechanism of further and finally destructive oxidation. The suggestion is invalidated by the observation that the addition of hydrogen peroxide does not influence the course of adrenaline auto-oxidation and that of catalase merely delays it (Fig. 8, curves 2 and 3).



Fig. 14.—Adrenaline oxidation in bicarbonate and phosphate buffers, alone and in the presence of equimolecular amounts of CuSO_4 . Final adrenaline and copper sulphate concentrations $\text{M}/2500$; pH 7.3; 37°C . Curve 1: Adrenaline in 0.016N bicarbonate in an atmosphere of 95 per cent. oxygen and 5 per cent. CO_2 .

Curve 2: Adrenaline in $\text{M}/15$ phosphate buffer.

Curve 3: Adrenaline plus copper sulphate in bicarbonate as in (1).

Curve 4: Adrenaline plus copper sulphate in phosphate as in (2).

(3) A different course of the reaction.—The early steps of adrenaline (I) oxidation are presented as proceeding from adrenaline to the unstable adrena-
linequinone (II), which quickly rearranges itself to leuco-adrenochrome (III),

this in turn reacting with further oxygen to form adrenochrome (IV). The redox potential of the leuco-adrenochrome/adrenochrome system is below that of the adrenaline/adrenaline-quinone system (Wiesner 1942; Falk 1949) (cf. Fig. 22). If during a very rapid enzymic oxidation *adrenaline-quinone is formed at a rate exceeding that of its rearrangement*, it would oxidize leuco-adrenochrome according to the equation in Figure 23 II from right to left. If this is the case, at no time would there be a noticeable concentration of leuco-adrenochrome in the reaction mixture. The suggestion that the presence of leuco-adrenochrome *and* adrenochrome is necessary for the onset of destructive oxidation is supported by the observation that, in adrenochrome catalysis, a time lag is observed (Figs. 12 and 13), indicating that a certain concentration of metabolites is necessary to establish the optimal rate of oxidation. Adrenochrome catalysis would thus seem to be lacking in the enzymic oxidation but to be an essential factor in the destructive oxidation of adrenaline*.

The structures of "green" and of "fluorescent" adrenochrome.—Marquardt (1947) formulated adrenochrome as a zwitterion, a *p*-quinone-immonium derivative (Fig. 16C): This structure is supported by the fact that only mono-substituted hydroxylamine, semicarbazone, and *p*-nitro-phenylhydrazine derivatives could be obtained (Green and Richter 1937; Braconier, Le Bihan, and Beaudet 1943). The synthesis of adrenochrome through oxidation of adrenaline by silver oxide or in presence of a polyphenolase justifies, however, the assumption of its basic structure as that of an *o*-quinone. Depending on the reaction to be interpreted, one or the other formulation may seem to be preferable; in the present paper the *o*-quinone formulae will be used for adrenochrome and oxo-adrenochrome (VI) (Fig. 22).

* It is possible that a similar mechanism prevails in the controlled oxidation of adrenaline to adrenochrome with oxidizing agents such as iodine, ferricyanide, or metallic ions in higher than catalytic concentrations; the reaction proceeds quickly and without any accumulation of intermediates by oxidation of the phenolic groups to adrenaline-quinone and adrenochrome. Chaix, Morin, and Jezequel (1950) comment on the fact that in bicarbonate buffer the oxidation by equimolecular proportions of cupric ion stops at the adrenochrome level while it exceeds it in phosphate buffer. They suggest that a complex is formed between adrenochrome and the phosphate ion; and assume further that this complex formation, by virtue of interference with phosphorylations, is responsible for the inhibition of glucose metabolism in tissue by adrenochrome. The oxidation of adrenaline by equimolecular or higher concentrations of oxidizing agents has not been examined in detail in the present investigation, but it would seem that the observation is equally well explained by the fact that adrenaline dissolves the (basic) copper carbonate formed in bicarbonate buffer while it does not, or only to a small extent, react with the tertiary copper phosphate formed in phosphate buffer. The unstable phenolic salts of copper would therefore be quantitatively formed in bicarbonate buffer and decompose quickly to quinones. In phosphate buffer the concentration of the phenolic salt would be less and the *primary* oxidation rate would be lower, leading to an accumulation of intermediates, to the onset of destructive oxidation, and therefore to a higher overall consumption of oxygen. Figure 16 illustrates the result of one experiment. In the interpretation of the results it must also be considered that acidic compounds are formed in the oxidation of adrenaline; the gas exchanges recorded cannot therefore simply be taken as oxygen uptakes. Cf. also the observation that the auto-oxidation of adrenaline starts earlier in phosphate-free medium (Holtz and Kroneberg 1950).

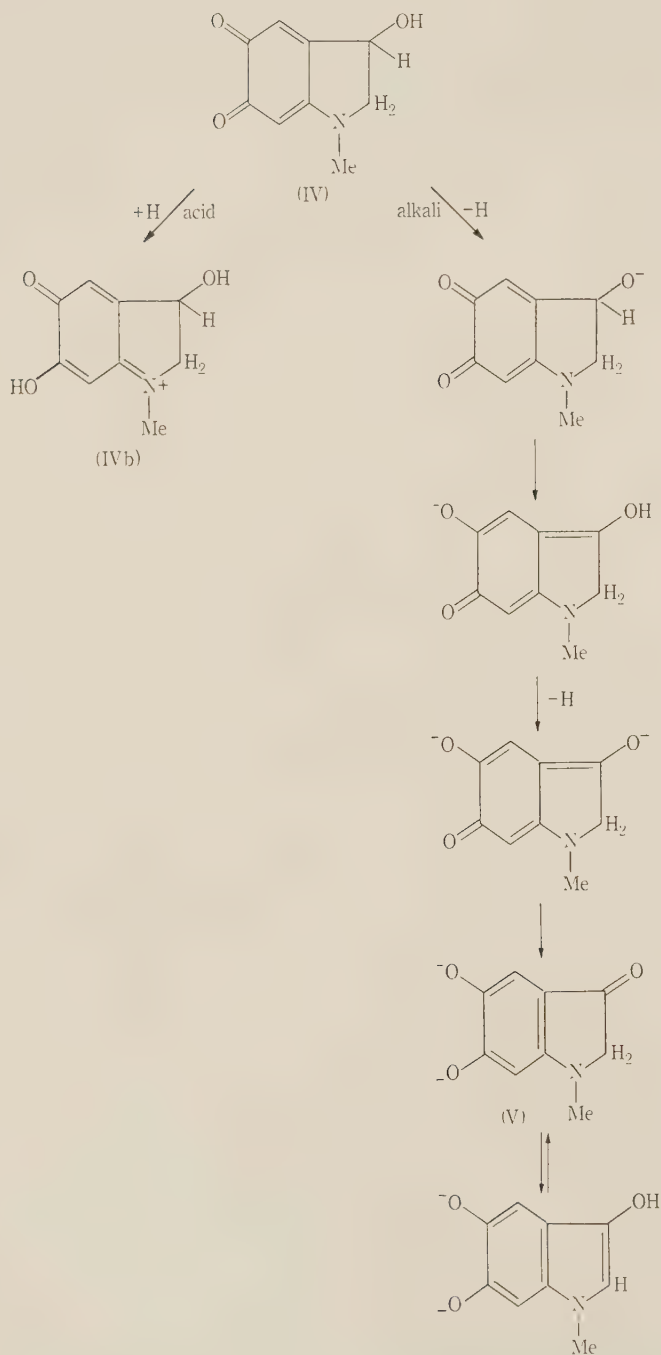


Fig. 15.—Formation of "green" and "fluorescent" adrenochrome by addition or loss of protons.

The following formulation is tentatively presented for the formation of "green adrenochrome" in acidic, and of "fluorescent adrenochrome" (N-methyl-5:6-dihydroxy-indoxyl) in alkaline medium. It is suggested that in acidic solution adrenochrome *adds one proton to a keto group of the o-quinone ring*, the positive charge of the proton being transferred to the nitrogen, forming

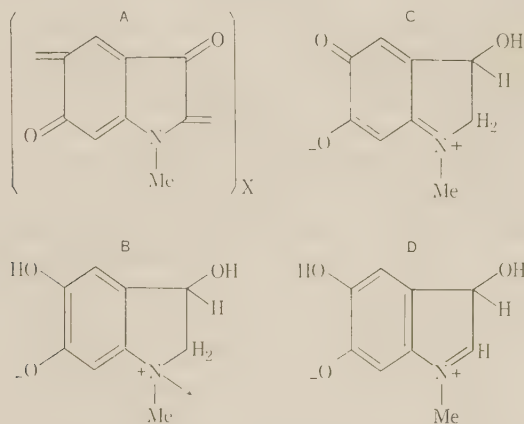


Fig. 16.—A: The structural element of melanin as proposed by Cohen (1945).

B: The semiquinone of the adrenochrome system as proposed by Harley-Mason (1948).

C: Zwitterion structure of adrenochrome.

D: Zwitterion structure of fluorescent compound as proposed by Harley-Mason (1948).

thus an immonium ion; only the structure in Figure 15 IVb is possible for this compound. Since the phenolic group cannot be supposed to be ionized in the acidic medium, the compound is not a zwitterion. It is suggested that in the acidic solution the remaining quinone oxygen reacts slowly with the $-\text{CH}_2-$ group activated by virtue of its position alpha to the immonium bond as a first step towards condensation. If the green colour observed is significant for this reaction, 30-60 minutes are required to complete it at room temperature. Neutralization at this stage no longer re-forms the red colour of adrenochrome. It is supposed that further oxidation takes place at the alcoholic group of the five-membered ring and that further condensation to chain structures occurs. On releasing the proton originally added, the positive charge on the nitrogen disappears and the condensation compound becomes insoluble, explaining the black precipitate formed within 24 hours.

In alkaline solution *adrenochrome loses two protons from the five-membered ring*; the negative charges thus created are transferred by electron rearrangement to the keto groups of the quinone ring, forming thus an ionized diphenol (Fig. 15 V). The transformation is almost instantaneous, and it is not reversible, since on neutralization or acidification the two protons lost are added *at the localization of the negative charges*: instead of adrenochrome the isomeric

4 : 5-dihydroxy-*N*-methyl-indoxyl is formed. On oxidation of the compound two electrons are removed from the ionized phenolic groups and oxo-adrenochrome is formed.

Since the successive removal of two protons first and then of two electrons constitutes only one oxidation step, the remarkable fact results that in the removal of two electrons and two protons from adrenochrome three distinct compounds figure in the reaction; the quinone adrenochrome, the diphenolic intermediate, and finally the keto quinone oxo-adrenochrome.

Melanin.—Bacq's statement, "The complete oxidation of adrenaline to melanin requires eight to nine atoms of oxygen per molecule of adrenaline and some CO₂ is evolved," citing Welsh (1934) as reference, is not correct. The analyses of the purest specimens of melanin indicate an oxidation level corresponding to the uptake of three atoms of oxygen, and the structure in Figure 16A has been proposed as the constituent unit of the molecule (Cohen 1945). This oxidation level is in agreement with the observation (in the trials of polyphenolase preparations) that the formation of a brown turbidity always indicated oxidation *beyond* the adrenochrome level even before the manometric readings could confirm it. The five to six more atoms of oxygen consumed by the solutions are therefore used in further and finally destructive oxidation of part of the adrenaline present. No conditions could be found that lead to a quantitative formation of melanin with an oxygen uptake of three atoms only; it would appear that melanin formation is a side reaction occurring during the slow destructive oxidation of adrenaline at physiological pH, and characteristic of it.

(c) *Fluorescence of Adrenaline Oxidation Products*

Adrenaline, especially in acidic solution, shows a slight fluorescence of its own. Loew (1918) was probably the first to describe the *strong* fluorescence observed during alkaline oxidation of adrenaline. West (1947), basing his work on that of Gaddum and Schild (1934) and others, presented an assay of adrenaline by the measurement of the maximum fluorescence obtained under standard conditions; Fischer and Lecomte (1949) used an almost identical procedure for the estimation of adrenochrome. Obviously the appearance of fluorescence is not specific for either substance. In fact, any adrenaline solution oxidizing at physiological pH gives, during the early stages of oxidation, fluorescence on alkalization, on alkaline oxidation, or on reduction. The present investigation is mainly concerned with the oxidation level of the fluorescent compounds and with the conditions under which red or fluorescent reaction mixtures are formed.

(i) *Fluorescence from Adrenaline*

At alkaline pH.—Figure 17 shows the fluorescence of M/2500 adrenaline with various concentrations of alkali under otherwise identical conditions. The maximum intensity of fluorescence is not obtained directly on admixture of the alkali; it develops slowly, especially with sodium carbonate or low concentration of free alkali. Carefully paralleled experiments suggest that the maximum

is reached not before the uptake of one atom of oxygen for one molecule of adrenaline and not later than corresponds to the uptake of two to three atoms. Then the intensity of fluorescence decreases and all fluorescence has disappeared when about six atoms of oxygen have been consumed.

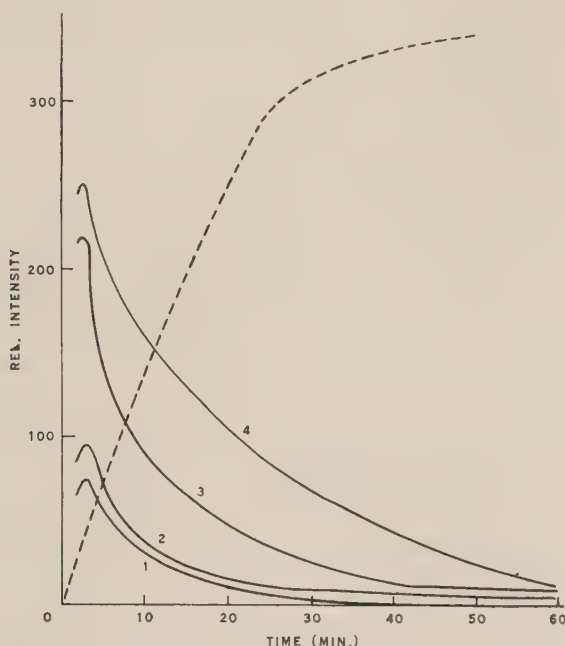


Fig. 17.—Adrenaline, fluorescence during alkaline oxidation. Final adrenaline concentration $M/2500$; 20°C .; tube frequently shaken to ensure adequate aeration.

Curve 1: Adrenaline in 0.5N sodium carbonate.

Curve 2: Adrenaline in 1.5N sodium carbonate.

Curve 3: Adrenaline in 0.5N caustic soda.

Curve 4: Adrenaline in 1.0N caustic soda.

The adrenaline oxidation curve in 1.0N caustic soda is superimposed in dots to illustrate the relation of fluorescence to oxygen uptake.

West (1947) proposed his assay for adrenaline concentrations of 10^{-6} - $10^{-7}M$, using sodium carbonate as the alkaline medium; the present authors could repeat his results. At higher adrenaline concentrations, however, it becomes difficult to maintain standard conditions since the intensity of the fluorescence depends not only on the HO^- ion concentration but also on the rate of aeration. If a solution of $M/500$ adrenaline, rendered alkaline with dilute sodium carbonate, is divided into three aliquots and air is excluded from the first by overlaying with paraffin oil, while the second is left standing and the third kept shaking, the fluorescence lasts longest in the first and least in the last tube; the peak intensity, however, is highest in the last and lowest in the first tube. It

is very doubtful whether the maximum intensities reached by different procedures are comparable and no attempt is made in the present work to draw any conclusions from slightly higher or slightly lower fluorescence intensities.

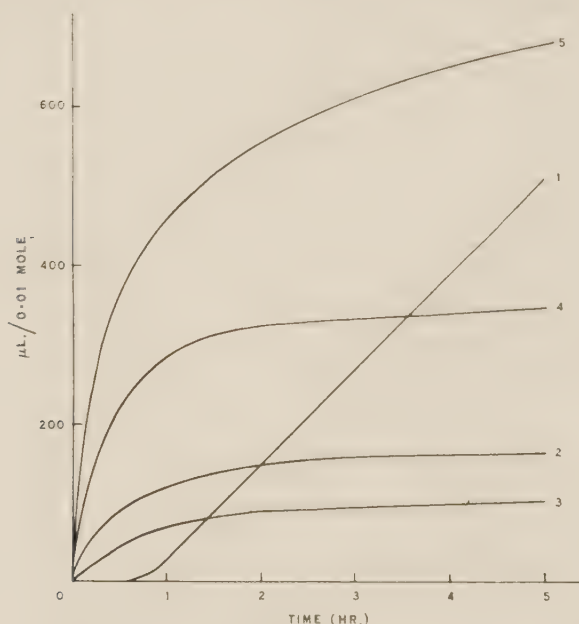


Fig. 18.—Adrenaline oxidation in the presence of sodium metabisulphite. Final adrenaline concentration $M/2500$, final sodium metabisulphite concentration $M/2500$, calculated as $Na_2S_2O_5$. $M/15$ phosphate buffer, pH 8.0; $37^\circ C$. The enzyme preparation showed no oxygen uptakes of its own. All reaction mixtures containing adrenaline and metabisulphate were colourless throughout the experiment but developed intensive green fluorescence.

Curve 1: Adrenaline alone.

Curve 2: Metabisulphite alone.

Curve 3: Adrenaline plus bisulphite.

Curve 4: Adrenaline plus 30 mg. enzyme and metabisulphite, pH 7.3.

Curve 5: Adrenaline plus 30 mg. enzyme, pH 7.3.

At physiological pH.—The auto-oxidation of adrenaline at physiological pH, catalysed or not, leads to coloured solutions. Fluorescent solutions are obtained only if the oxidation is conducted in the presence of a reducing agent.

If mixtures of adrenaline and sulphite are allowed to react in a Warburg manometer, the mixtures consume less oxygen than the sum of the two components singly reacted under the same conditions (Fig. 18); in the mixture the components show a "mutual inhibition of oxidation." At neutrality the rate of oxygen uptake is low and no visible changes may be observed for a con-

siderable time; at a slightly higher pH (8-9) *strongly fluorescent, colourless* solutions are obtained. The oxygen uptake quickly exceeds one atom of oxygen for one molecule of adrenaline but then the curve levels out and flattens between the levels of one and two atoms. The further oxidation proceeds very slowly and the intensity of fluorescence remains almost constant for a considerable time (cf. Shaw's (1938) adrenaline assay). The same result can be observed if adrenaline is allowed to oxidize slowly in the presence of glycine.

At more neutral pH the onset of adrenaline oxidation in the presence of sulphite can be accelerated by a polyphenolase. The reducing agent does not appear to affect the action of the enzyme seriously; an initial rapid uptake of slightly over two atoms for one molecule of adrenaline is observed, but then the curve flattens out exactly as in the previous case (Fig. 18, curve 4). The solutions remain throughout completely colourless and strongly fluorescent and at no time is the red colour of adrenochrome observable. The maximum intensity of fluorescence is reached with the uptake of slightly over two atoms of oxygen and remains almost unchanged for several hours.

(ii) *Fluorescence from Adrenochrome*

On reduction.—Adrenochrome (or any of the coloured solutions in the early stages of adrenaline oxidation) is readily reduced to fluorescent solutions; almost any form of reduction leads to some fluorescence, but, since adrenochrome is unstable at acidic or alkaline pH, the reaction is best conducted at about neutral pH. Ascorbic acid, sulphite, bisulphite, metabisulphite, and other hydrogen donors of low redox potential—but not thiosulphate—give almost immediate discoloration. If pure adrenochrome in concentrations of M/2000 or less is used, the resulting solution is absolutely colourless and strongly fluorescent (Fig. 20, curve 2); it is moderately stable in the presence of the reducing agent but begins to decompose if the latter is removed.

The reaction is reversible. Iodine in potassium iodide is a suitable reagent to remove excess of sulphite; if it is added drop by drop, the end point of the sulphite titration is reached when the brown drops of iodine become surrounded by a large red halo before their colour finally disappears. If more iodine is added, adrenochrome is re-formed; excess iodine may be removed by thiosulphate. If care is taken to keep the reaction mixture at neutral pH, the reduction and re-oxidation may be repeated two or three times before the solution becomes too dilute and side reactions remove the reactants. For purposes of physiological investigation of the fluorescent compound, dimercaptopropanol (BAL) is a good reducing agent for adrenochrome since in the concentrations needed neither BAL nor its oxidation products are physiologically active.

On alkaline oxidation.—Figure 19A shows the fluorescence measured on treating M/2500 adrenochrome with varying concentrations of alkali, and Figure 19B some results obtained with adrenochrome dilutions of 10^{-5} , the concentration used by West for his assay of adrenaline. Old or too concentrated adrenochrome solutions incline to form a fluorescence with a brown or orange tint, with

pure adrenochrome the solutions are *yellow* and identical with those obtained under the same conditions from adrenaline. The maximum intensities observed are slightly higher than those given by the same concentrations of adrenaline. The differences are: with adrenochrome the peak intensity is reached almost immediately on admixture, and the duration of the fluorescence is shorter than with adrenaline.

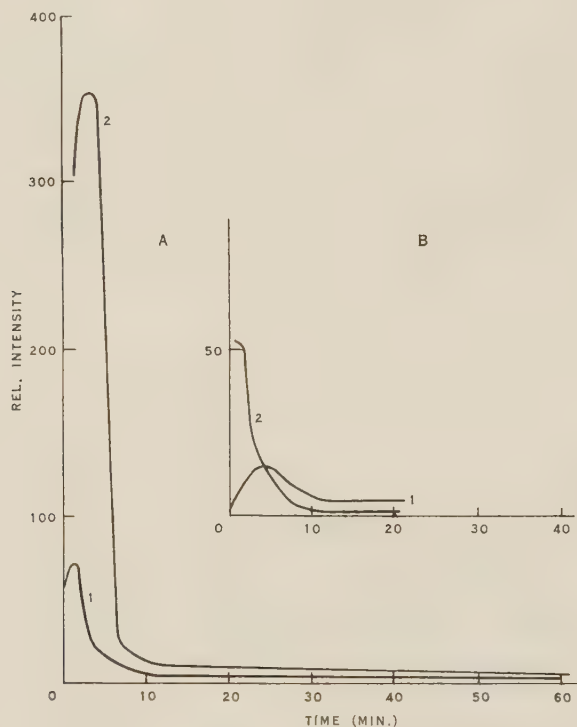


Fig. 19.—Adrenochrome, fluorescence during alkaline oxidation. A: Final adrenochrome concentration M/2500; 20°C.; tubes shaken to ensure adequate aeration.

Curve 1: Adrenochrome in sodium carbonate 1.5N.

Curve 2: Adrenochrome in 1N caustic soda.

B: Final adrenochrome concentration M/100,000.

Curve 1: Adrenochrome in 1.5N sodium carbonate.

Curve 2: Adrenochrome in 1N caustic soda.

The fluorescence formed on alkaline oxidation is of higher intensity than that formed on reduction. The addition of alkali to reduced adrenochrome further increases the fluorescence (Fig. 20, dotted line).

(iii) *Reactions of the Fluorescent Solutions*

The reactions given are the same as those described for the early yellow-fluorescent solutions obtained on the alkaline oxidation of adrenaline: positive

tests for the catechol grouping and for the indole or indoxyl structure. These reactions are given whether the fluorescence has been formed by reduction, oxidation, or, as with "fluorescent adrenochrome," without admission of air. The solutions are moderately stable at neutral or slightly acidic pH, but decompose on attempts at separation of the fluorescent compounds, as well as on prolonged standing. They oxidize faster than adrenaline. The oxidation to red quinones is not always possible if the solutions are aged; it would appear that further decomposition of the compounds takes place without the formation of quinones and melanins. The composition of these final yellow-brown solutions has not been investigated.

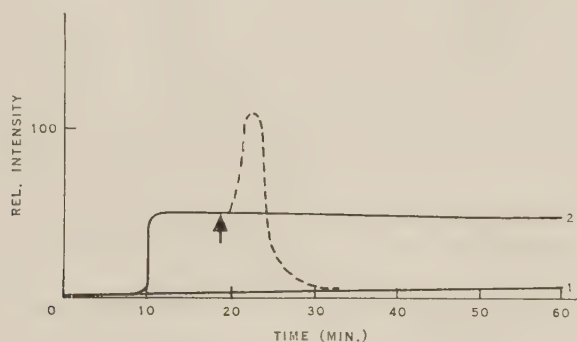


Fig. 20.—Adrenochrome, fluorescence with sodium sulphite. Final adrenochrome concentration $M/2500$; 20°C .; tubes shaken to ensure adequate aeration. Initial medium $M/15$ phosphate buffer, pH 7.0; sulphite concentration $M/10$.

Curve 1: Adrenochrome alone.

Curve 2: Adrenochrome plus sulphite; caustic soda, 1 ml. 1N, added at arrow.

IV. CONDITIONS OF FORMATION OF FLUORESCENT OR RED SOLUTIONS

(a) At Weakly Alkaline pH

If a dilute solution of adrenaline is rendered weakly alkaline by the addition of a few drops of $N/10$ KOH, it turns quickly red, consuming the oxygen dissolved in the liquid. After a few seconds, however, the colour fades and a yellow, fluorescent solution remains. On shaking with air, the red colour reappears, but fades again on standing. The procedure can be repeated three or four times, but then no more red colour is formed on shaking, nor is there any fluorescence left in the slightly yellow solution. The experiment, recorded by Loew (1918), is a modification of the repeated reduction and re-oxidation of adrenochrome just described, except that neither reducing nor oxidizing agents have been added. The oxidation of adrenaline to the red quinones is caused by more intensive aeration and the reduction of the quinones is brought about through the transfer of hydrogen to them from intermediates in the later stages of the reaction sequence. Figure 21 shows the fluorescence measured

during an experiment. It is seen that the intensity of fluorescence drops when the red compounds are formed on aeration, and increases again when they are reduced on standing, though not to the previous level, nor to the level of an identical solution that has not been shaken. After repeated shakings the stock of quickly reducible or oxidizable compounds becomes exhausted and the solutions no longer give catechol or indole reactions. The experiment succeeds equally well in barium hydroxide solution.

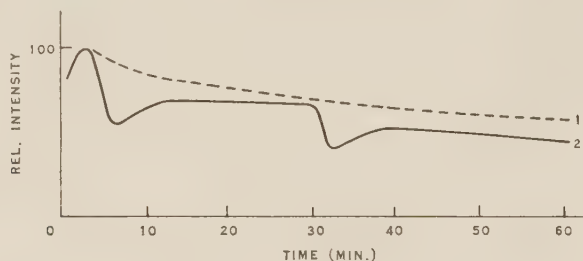


Fig. 21.—Adrenaline, fluorescence with different rates of aeration. Final adrenaline concentration $M/2500$; 20°C .

Curve 1: Adrenaline in $1.5N$ sodium carbonate, tube not shaken.

Curve 2: Same, but tube strongly shaken at 3 and 30 min.

A distinct pink colour was observed on shaking while the intensity of the fluorescence decreased, and disappeared when the fluorescence began to increase again.

(b) At Physiological pH

If adrenaline is allowed to oxidize under reduced oxygen supply, e.g. by overlaying the solution with a thin layer of paraffin, the formation of an early fluorescence is observed; but the reaction proceeds very slowly and the solutions soon become turbid and brownish. If the first steps of the reaction are accelerated, e.g. by placing a slice of dahlia tuber in the bottom of the test tube, a strongly fluorescent solution with a thin, red top layer is quickly obtained. On slight shaking, the whole liquid becomes temporarily red, but loses its colour again on standing. The final solutions are non-fluorescent, light brown, and slightly turbid, but considerably lighter in colour than the dark red-brown solutions obtained with intensive aeration. The oxygen uptakes under these conditions are difficult to follow.

(c) Redox Potentials

An attempt was made to determine the redox potential of the quinonoid system. Reliable values are difficult to obtain owing to the inherent instability of the system; the solutions undergo a continuous change towards the final destructive oxidation of adrenaline. The adrenaline/adrenaline-quinone potential, established by Ball and Chen (1933) by measurement at acidic pH, is taken

to be at neutrality only slightly lower than that of catechol/*o*-quinone, about +0.38 volts referred to the normal hydrogen electrode. Falk (1949), quoting Wiesner (1942), places the leuco-adrenochrome/adrenochrome potential at about +0.04 volts, slightly above that of methylene blue/leuco-methylene blue. Attempts were made to measure potentiometrically the redox potential of very pure adrenochrome solutions by treating them with increasing quantities of mild reducing agents like BAL or ascorbic acid. No complete titration curves could be obtained, but the readings at the start of the titration seem to indicate values about 0.1 volt below the potential of the quinhydrone electrode, between +0.1 and +0.2 volts. This value is higher than Falk's, but it appears to be in better agreement with observations made with leuco-methylene blue.

(d) Action of Leuco-Methylene Blue

Methylene blue itself, at physiological pH, has no quick action on either adrenaline or adrenochrome; the mixtures are blue with adrenaline, or violet with adrenochrome, and turn dirty violet or dirty brown as the atmospheric oxidation proceeds. If, however, a solution of adrenochrome, or any early red solution obtained on adrenaline oxidation, is treated with leuco-methylene blue, the red colour fades instantaneously, the mixture turns blue and strongly fluorescent, showing that *all quinones present have potentials sufficiently above that of methylene blue/leuco-methylene blue to allow a quick and quantitative reaction*. The further changes are slow at physiological pH. If the oxidation rate of the fluorescent compounds is accelerated by the addition of a few drops of N/10 KOH, the blue colour of methylene blue fades and a yellow-brown, *fluorescent* solution is formed that is slightly turbid through the precipitation of some leuco-methylene blue. The top layer in contact with air shows a thin but distinct blue zone; on shaking with air some methylene blue is re-formed but the colour fades again on standing. Under the conditions of the experiment methylene blue acts as oxidation mediator exactly as the quinone system in the experiments previously described. After 12-24 hours the blue colour of methylene blue begins to reappear and remains, though with low intensity since most of the dye has been decomposed. Methylene blue is reduced only while the oxidation of adrenaline proceeds at a noticeable rate, indicating the temporary presence in the oxidizing mixture of an effective concentration of compounds of a redox potential lower than that of methylene blue under the conditions. The final yellow solutions obtained on alkaline oxidation no longer react with methylene blue.

V. DISCUSSION AND CONCLUSIONS

(a) The Redox Systems in Adrenaline Oxidation

(i) *Nature of the Fluorescent Compounds*.—By their reactions the fluorescent compounds are to be considered as *o*-diphenols possessing indole or indoxyl structures, representing the reduction products of the corresponding red *o*-

quinones. Utevski (1944) and West (1947) proposed the fluorescent compound to be leuco-adrenochrome, corresponding to an oxygen uptake of *one atom of oxygen* for one molecule of adrenaline, or to the reduction product of adrenochrome formed by the uptake of two hydrogens and two electrons at the

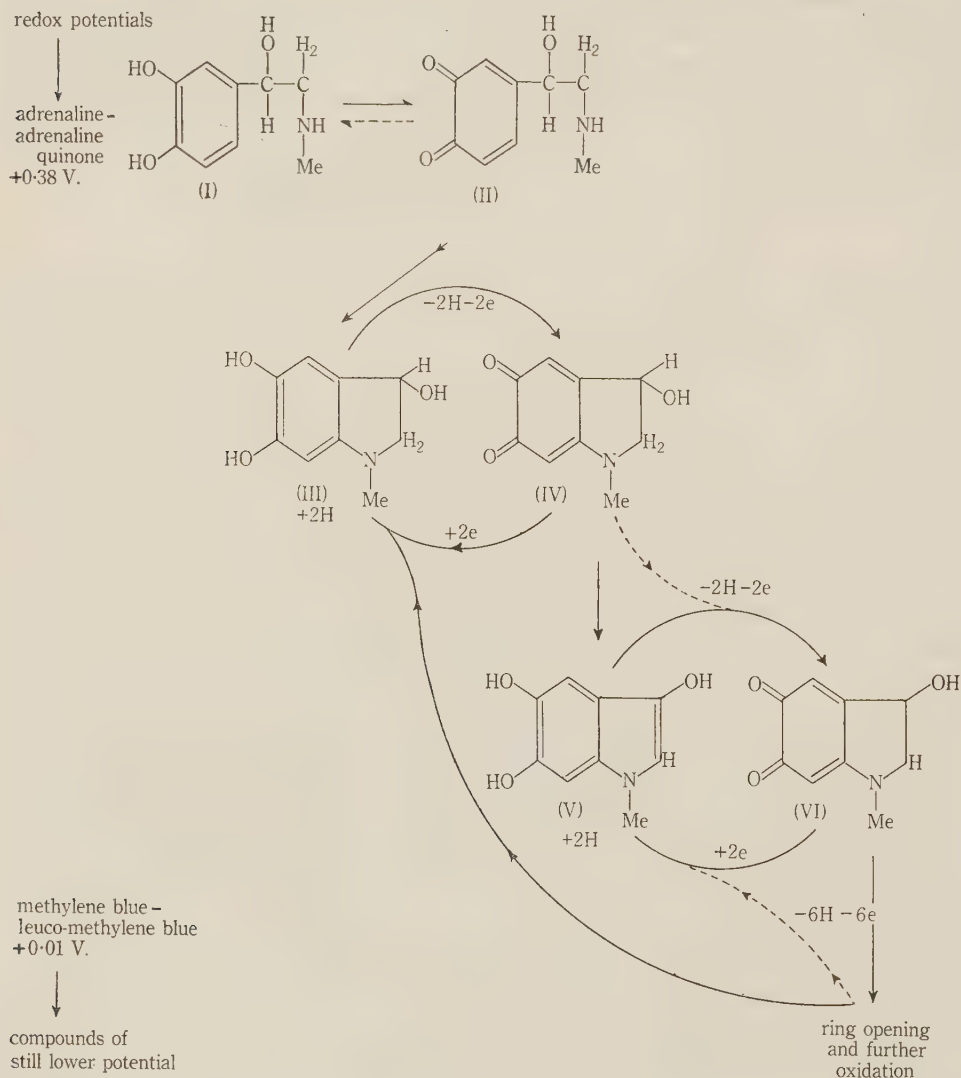


Fig. 22.—Redox systems in adrenaline oxidation.

quinone grouping. They state that the compound is fairly stable at pH 6-7, although decomposing on attempted isolation. Falk (1949) also considered leuco-adrenochrome to be the fluorescent compound, but assumed that it is rapidly oxidized by air at physiological pH. Harley-Mason (1948) claimed that leuco-adrenochrome is so unstable that it could not be formed during adrenaline oxidation since one should never obtain adrenochrome from it. He proposed

the fluorescent compound to be an isomer of adrenochrome, corresponding therefore to the uptake of *two atoms of oxygen* for one molecule of adrenaline and ascribed to it the zwitterion structure (Fig. 16D). Such an isomer has been obtained by Lund (1949) by controlled alkaline oxidation of adrenaline; the compound was separated in *yellow-green* crystals that gave fluorescent solutions and the catechol and indole reactions mentioned. Its composition was ascertained by analysis. In as yet unpublished work, Harley-Mason proposed the compound to be *N*-methyl-5 : 6-dihydroxy-indoxyl, the leuco form of oxo-adrenochrome (Fig. 22 VI) synthesized by Cohen (1945).

The present authors believe, however, that this compound is not the only fluorescent compound formed during adrenaline oxidation. They are of the opinion that the evidence presented by Harley-Mason proves merely the limited stability of leuco-adrenochrome, but does not disprove the possibility of its initial formation nor of its temporary role during the atmospheric oxidation of very dilute adrenaline solutions. The formation of *colourless* fluorescent solutions with oxygen uptakes of little over one atom for one molecule of adrenaline, and the reduction and re-oxidation of adrenochrome as described in this paper are accepted as demonstrating the existence of leuco-adrenochrome as a fluorescent oxidation intermediate. In the following discussion the fluorescent compounds as a group will be referred to as diphenols in relation to their oxidation products, the red quinones.

(ii) *Conditions of Formation of Fluorescent Di-phenols and of Red Quinones.*—It may be surprising that during the intensive oxygen uptakes of alkaline oxidation of adrenaline a very noticeable amount, if not the bulk of the reactants, is present in the *reduced* form as fluorescent diphenols, while during the slow oxidation at lower pH the bulk of the reactants is present in the form of the *oxidized* red quinones and their condensation product, melanin. Loew's experiment shows that in the same weakly alkaline solution fluorescent diphenols or red quinones can be observed *dependent on the rate of aeration*. It is suggested that the question of whether the solution contains the red quinones or the fluorescent diphenols depends on the relative rates of primary oxidation and of dehydrogenation. Under conditions of strongly alkaline oxidation the tendency of later oxidation products to transfer hydrogen to the quinones is so strong that only the reduced forms, namely the diphenols, are present, forming yellow, strongly fluorescent solutions; at lower pH the reactions of dehydrogenation proceed but slowly and the solution becomes enriched in the oxy forms, namely the red quinones, to a degree that even allows some condensation, red-brown, turbid, melanin-containing solutions being formed. If the intensity of hydrogen transfer is increased, e.g. by the addition of sulphite, glycine, or serum, fluorescent solutions are obtained.

These differences in the visible changes between oxidation at physiological and at more alkaline pH need therefore not necessarily indicate a fundamental difference in the mechanism of oxidation; in both cases the same end products

may be formed. At lower pH the red quinones accumulate in the solution, at higher pH they are rapidly dehydrogenated by the products of further oxidation.

(iii) *Redox Systems*.—It is suggested that the constant oxidation mechanism, indicated by the constant oxidation rate prevailing during the later phases of adrenaline oxidation at physiological pH, functions over redox systems that act as oxygen consumers and mediate the oxidation of later reaction products by accepting hydrogen and electrons. Owing to the instability of adrenaline-quinone it is unlikely that the adrenaline/adrenaline-quinone system plays a major role—with the possible exception considered in discussing the oxidation of adrenaline by a very pure polyphenolase. The first redox system formed would therefore be the leuco-adrenochrome/adrenochrome system. The functioning of adrenochrome as a hydrogen acceptor for malic and lactic dehydrogenases (Green and Richter 1937) proves the system to be fairly stable. Its action, under physiological conditions has been suggested, e.g. for the synthesis of acetyl-choline in brain tissue (Torda and Wolff 1946) or in isolated intestine (Minz and Plotka 1947). It is considered that, during the early phases of adrenaline oxidation, leuco-adrenochrome is formed and acts as the reductant of the adrenochrome system; later in the reaction sequence the role may be shared or taken over by the *N*-methyl-5 : 6-dihydroxy-indoxyl/oxo-adrenochrome system. Figure 22 presents a scheme of the quinonoid systems together with such indications to redox potential as appear to be justified. It must be noted that:

(1) Although some of the later oxidation products may preferably transfer hydrogen to a suitable acceptor, quinone, or methylene blue, there is no evidence that the primary oxidation mechanism is confined to the re-oxidation of the diphenols. Some oxidation intermediates may react directly with oxygen;

(2) The visible changes observed during the oxidation of adrenaline show the presence of red quinones or of fluorescent diphenols; this observation does not exclude the functioning of other than quinonoid redox systems, say of indoxyl or dioxindol structures.

(b) *The Mechanism of Destructive Adrenaline Oxidation*

(i) *Onset of Oxidation*

Auto-catalysis and metal catalysis.—Adrenaline in an ideal pure solution at neutral pH would possibly be indefinitely stable; actually oxygen uptakes become noticeable *after a time lag* of several hours. It is accepted that the onset of oxidation is due to traces of metallic ions present in the solution (or, at more elevated pH, to HO ions). The action of these ions can be assumed to consist in the formation of unstable adrenaline ions, which readily lose electrons, adrenaline-quinone being formed in the process. The overall reaction is presented in Figure 23 I. The electrons liberated either reduce the metallic ions or, at higher pH, react directly with atmospheric oxygen. The adrenaline-quinone rearranges itself to leuco-adrenochrome, which in turn is oxidized by

the same mechanism, though at a faster rate, to adrenochrome. The latter accumulates in the solution which, towards the end of the time lag, acquires a faint pink tint. Higher concentrations of catalyst, metallic or HO ions, or higher temperature shorten the time lag.

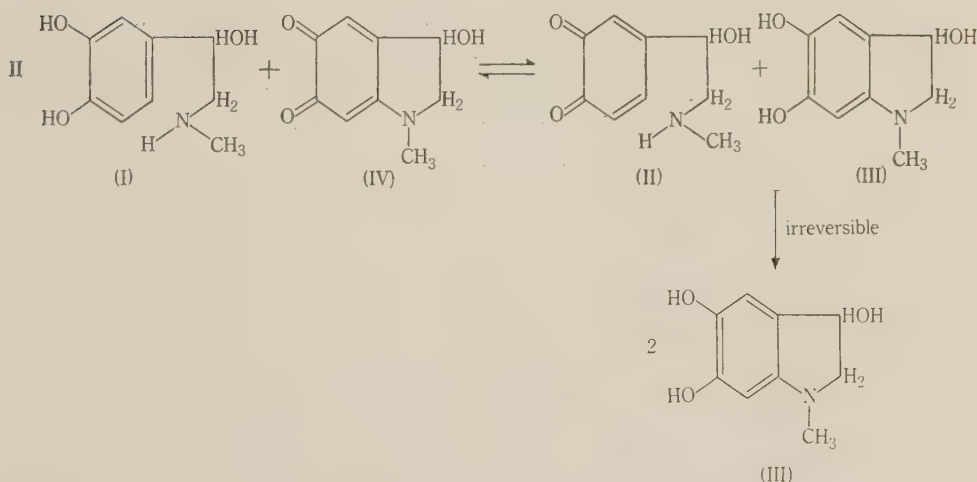
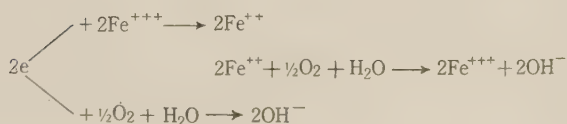
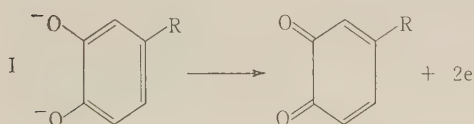


Fig. 23.—I. Mechanism of metal and HO ion catalysis.

II. Mechanism of adrenochrome catalysis.

Adrenochrome catalysis.—A different mechanism must be considered to explain the fact that the addition of small amounts of adrenochrome leads to exactly the same shortening of the time lag. It is suggested that the redox potentials of the adrenaline/adrenaline-quinone system and of the leuco-adrenochrome/adrenochrome system are near enough to each other for the reductant of higher potential, namely adrenaline, to be oxidized by the oxidant of lower potential, namely adrenochrome (Fig. 23 II from left to right); adrenaline-quinone and leuco-adrenochrome are formed in the process, or—owing to the quick rearrangement of the former—two molecules of leuco-adrenochrome. The

latter is oxidized at a faster rate than adrenaline and the sluggishly oxidizable adrenaline can be considered as being "mobilized" by the action of adrenochrome and in proportion to the adrenochrome concentration reached. Since adrenaline-quinone is short-lived, the redox potential of the adrenaline system will remain low, while that of the adrenochrome system remains high as long as the leuco-adrenochrome formed is oxidized at the rate of its formation. The solutions will contain unreacted adrenaline and adrenochrome without any effective concentrations of adrenaline-quinone or leuco-adrenochrome, i.e. of redox systems.

Chemical mechanism during the time lag.—It is suggested that the auto-oxidation of adrenaline at physiological pH is *started* by such traces of metallic ions as are present in the solution and then *further promoted* by adrenochrome catalysis. The oxidation rate remains low as long as the solutions contain adrenaline and adrenochrome alone, but the conditions change when the capacity of the primary oxidation system is reached and leuco-adrenochrome begins to accumulate in the solution.

(ii) *The Period of Rapid Acceleration*

Almost abruptly the oxygen uptakes begin to increase and the colour of the solution intensifies from pink to red. This period of acceleration is of short duration, about half an hour, and terminates in a steady, almost constant oxidation rate. It is tentatively proposed that the acceleration is due to the formation of more readily oxidizable compounds.

The onset of the acceleration.—The onset of acceleration is hastened by the addition of catalysts, but it is only little affected by the concentration of adrenaline (Fig. 3). It seems to coincide with the moment when in an unbuffered solution the pH begins to drop and appears to depend on the oxygen uptakes which, under the conditions of auto-oxidation, amount to about one-fourth atom of oxygen for one molecule of adrenaline. This uptake would correspond to the oxidation of about 5 per cent. of the adrenaline present to adrenochrome. Since the addition of this or a slightly higher proportion of adrenochrome just causes the time lag to disappear (Fig. 5, curve 3), it would seem that at these proportions the final oxidation mechanism is established without much delay.

The formation of more readily oxidizable compounds.—Indoxyl in alkaline solution is quickly oxidized by atmospheric oxygen. The oxygen substitution in the benzene ring will certainly not lower the oxidizability of the compound and it is likely that the formation of indoxyl structures is the critical step towards the further oxidation of adrenochrome. The formation of *N*-methyl-5:6-dihydroxy-indoxyl from adrenochrome in alkaline solution has been discussed. It is suggested that at physiological pH the transition takes place *in the mixture of leuco-adrenochrome and adrenochrome*, the overall reaction consisting of the oxidation of the dihydroindoxyl grouping of leuco-adrenochrome by the quinone grouping of adrenochrome. The mechanism of this step involves probably the disproportionation of free radicals. Oppenheimer and Stern (1939) reviewed evidence that the action of adrenochrome as hydrogen acceptor, e.g. in

the oxidation of amino acids, proceeds in a univalent reduction to a semiquinone. Harley-Mason (1948) found that in the reduction of adrenochrome by hydrogen in the presence of palladium-charcoal only one atom of hydrogen is consumed per molecule of adrenochrome and equimolecular proportions of *N*-methyl-5 : 6-dihydroxy-indole and *N*-methyl-5 : 6-dihydroxy-indoxyl were isolated from the reaction mixture. Harley-Mason proposed for the semiquinone formed the zwitterion structure in Figure 16 B with the unpaired electron at the positively charged nitrogen atom.

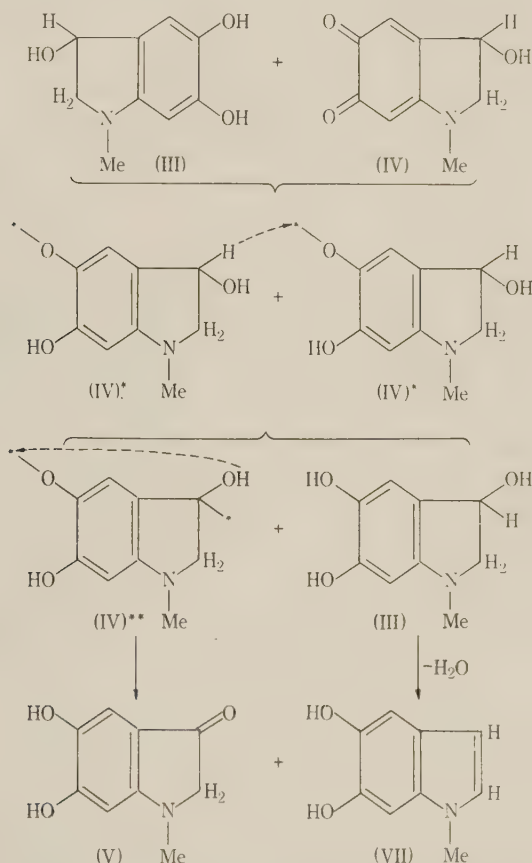


Fig. 24.—Free radical mechanism of the transition from the adrenochrome to the indoxyl structure (explanation in text).

A different formulation, for which the authors acknowledge the helpful advice of Dr. K. H. Pausacker,* and a tentative mechanism of the reaction sequence are presented in Figure 24. In the formation of the semiquinone (IV*) the hydrogen becomes attached to the 6 oxygen which, as shown by the possibility of a zwitterion structure for adrenochrome, has the higher electronegativity. The unpaired electron attaches itself to the remaining 7 oxygen.

* Department of Chemistry, University of Melbourne.

This free radical abstracts from the dihydroindoxyl grouping of another radical the 3 hydrogen atom, which is reactive through its position next to the HO group. In this disproportionation one molecule of leuco-adrenochrome is formed and one of the hypothetical highly unstable di-radical (IV^{**}), which rearranges itself to the stable *N*-methyl-5 : 6-dihydroxy-indoxyl. It is supposed that under the conditions of auto-oxidation at physiological pH the leuco-adrenochrome formed returns into the adrenochrome redox system, while the indoxyl compound undergoes rapid further oxidation. The immediate effect of the reaction shows itself in increased oxygen uptakes leading to further oxidation and to the onset of destructive oxidation, with the formation of brown condensation products and dicarboxylic acids or other more acidic compounds. If the oxidation is slow, or on attempts at concentration and separation of the compounds further changes take place, e.g. condensations and other reactions, such as the dehydration of leuco-adrenochrome to *N*-methyl-5 : 6-dihydroxy-indole (VII).

(iii) *The Period of Constant Oxidation Rate*

The onset of constant oxidation.—The period of acceleration terminates under the conditions of atmospheric oxidation with the uptake of about half an atom of oxygen for one molecule of adrenaline, corresponding to the oxidation of about 12 per cent. of the adrenaline present to adrenochrome level.

(1) *The role of the catalysts.*—The oxidation rate finally established is the same within a wide range of catalyst concentrations (Fig. 4; also Falk 1949; Slater 1949, for haematin and methaemoglobin catalysis), and there is no evidence that the nature or amount of the catalyst plays any part in the course of the later oxidation. Da Fonseca-Ribeiro and Cardoso (1949) report that oxalic acid inhibits the auto-oxidation of adrenaline and suggested that salt formation between the acid and the metal ions competes with the formation of the phenolic salt. The observation could be confirmed and it is tentatively considered that early oxidation products after ring opening, possibly dicarboxylic acids, combine with the metallic ions, possibly by chelation, and prevent their further catalytic function (cf. also the inhibition of copper catalysis by complex formation with glycine (Verly 1948)). If this is the case, the reaction mechanism establishing and controlling the constant rate of oxidation would rest entirely between adrenaline and its oxidation products and be the same whichever way the period of acceleration was reached. This assumption is in agreement with the observation that adrenochrome catalysis also leads to the same final rate of oxidation (Fig. 5).

(2) *The role of adrenaline concentration.*—The constant rate of oxidation is maintained for several hours, from the time when about 80 per cent. of the adrenaline originally present must still be unreacted up to the uptake of 4.5 atoms of oxygen when at least 80 per cent. of the adrenaline must be accepted as being oxidized beyond opening of the ring structure. Evidently the later concentration of adrenaline is not one of the factors which regulate the oxidation rate. The final rate, however, appears to depend on the *original* concentration of adrenaline insofar as the same molar oxidation rate is reached (Fig. 3).

Maintenance of the oxidation rate.—On these observations it would appear that the mechanism of constant oxidation is *established* when a certain proportion of adrenaline to its oxidation products (corresponding to *c.* 12 per cent. adrenochrome) is reached, but that from then on adrenaline becomes merely the substrate of an oxidation mechanism that functions steadily, at full capacity and at a constant rate practically to the time when the stock of unreacted adrenaline becomes exhausted. It is suggested that two factors maintain the oxidation rate; the concentration and redox potential of the leuco-adrenochrome/adrenochrome system, and the retarding influence of later oxidation products.

(1) The redox potential.—The process of further and finally destructive oxidation proceeds in a series of steps following each other until the final products have no longer any efficient tendency to lose their hydrogen. Accepting adrenochrome as the critical level beyond which non-arrestible further oxidation starts, four to six atoms of oxygen are consumed by each molecule on its way. Even if only a fraction of this oxidation takes place over redox systems, such an intensive transfer of hydrogens must considerably increase the concentration of diphenols, mainly leuco-adrenochrome. This effect lowers the redox potential of the leuco-adrenochrome/adrenochrome systems and therefore decreases the rate of adrenaline mobilization on the mechanism suggested (Fig. 23 II from right to left); it leads also to a lowering of the concentration of the redox system which is depleted by destructive oxidation, but not replenished by adequate oxidation of adrenaline. The reaction mixtures in question are too turbid and complex to allow quantitative examination, and, without knowing the nature, redox potential, and reactivity of each intermediary it is impossible to outline the exact mechanism of the reaction sequence. It can, however, be understood that the reaction will stabilize itself at a state in which adrenaline enters the quinonoid systems at the same rate at which other molecules leave it.

(2) The role of later oxidation products.—In the presence of a moderate excess of metallic catalyst there occurs between the time lag and the period of final constant oxidation a period of steady, more rapid oxidation, the duration of which depends on the amount of catalyst added (Fig. 4). It is reasonable that excess of the catalyst constitutes a more efficient oxidizing system and leads to the rapid formation of a higher concentration of early oxidation products, mainly adrenochrome, and that the addition of adrenochrome itself has the same effect (Fig. 5). It is, however, difficult to see why the high oxidation rate, once established, should be of limited duration and eventually drop to about the same value that would have been reached without the addition of any catalyst. It is suggested that the solutions at this stage can be considered to be identical whichever way the high adrenochrome concentration has been affected: they contain *more adrenochrome* than would have been formed under the conditions of uncatalysed auto-oxidation and it would appear as if the excess adrenochrome forcibly formed were consumed before the "normal," final oxidation rate is established.

Ascorbic and pyruvic acids form mixtures with adrenaline that are very resistant towards oxidation. Obrecht (1939) proposed a mathematical formulation of the effect and Verly (1948) and Marquardt (1947) discussed it further. These mixtures consume less oxygen than the components singly, indicating that the tendency to react with atmospheric oxygen is considerably lowered. A late oxidation intermediary of adrenaline, keto acid, or other, may in the same way cause a decrease of "oxidizability" showing itself on all diphenols, but mainly on that of the highest potential, namely adrenaline. The effect would be that the bulk of adrenaline is preserved, and the components of the quinonoid systems are consumed until the concentration of the inhibiting intermediate, formed by a diminished amount of quinonoid systems, is low enough to allow further oxidation of adrenaline itself.

The sequence of acceleration and destructive oxidation.—If this presentation is accepted, there should be a *time interval* between the onset of the two reactions: First an increased oxidation rate is caused through the formation of indoxyl structures and their further oxidation to dioxindol and isatin structures; later, when the ring structures are opened, dicarboxylic acids are formed and inhibit the course of the earlier reaction in the manner discussed. Under suitable conditions, e.g. in the oxidation of adrenochrome alone, the first reaction, acceleration, overshoots the steady oxidation rate demanded by the conditions (Fig. 11); the final regulation is effected when, later on, the mechanism of destructive oxidation has become established. It is possible that the temporary increase of the early oxidation rate suggested in the oxidation of adrenaline-adrenochrome mixture (Figs. 5 and 12) represents also this phase of "overshooting," and it is suggested that the effect of excess of metallic catalyst (Figs. 4 and 14) is due to the same cause. In the oxidation of adrenaline alone this early "hump" cannot be expected since the concentration of adrenochrome increases so slowly that all mechanisms concerned can keep in step; the optimum oxidation rate is directly reached and then maintained.

(c) Practical Applications

(i) *Adrenaline Assay.*—West's assay of adrenaline (1947) by determination of the maximum intensity of the fluorescence formed under standard conditions of alkaline oxidation appears to be reliable for the dilutions proposed (10^{-6} - 10^{-7} M) so long as it is ascertained that the unknown specimen contains adrenaline only. With higher concentrations of adrenaline the development of fluorescence depends too much on the conditions of the experiment to ensure reliably constant conditions. Furthermore, the fluorescence formed from early adrenaline oxidation products, viz. adrenochrome, is of the same order of intensity as that of adrenaline itself. The assay is not specific.

The colorimetric assay by measuring the adrenochrome colour formed on oxidation with iodine followed by destruction of the excess iodine with thio-sulphate (Evans and Raper 1937) suffers from the same disadvantage since leuco-adrenochrome also is oxidized to adrenochrome by the same procedure. Its presence may, however, be detected by the fact that the solution shows a

noticeable fluorescence; adrenaline itself is only slightly fluorescent under the conditions. A further disadvantage is that the colour of adrenochrome is unstable, melanin condensation taking place very quickly.

The oxygen uptakes at physiological pH will only demonstrate the presence of a steadily functioning redox system; for estimation of the concentration of adrenaline or its lower oxidation products they are valueless, especially if other reductants, amino acids, etc., are present that may react with the system. The initial rapid oxygen uptakes at strongly alkaline oxidation (six atoms for one molecule of adrenaline, four for one of adrenochrome) measure possibly the oxygen required for the opening of the ring structures and may occasionally give confirmatory evidence as to the content of a mixed solution.

The formation of a strong fluorescence on anaerobic alkalization may reveal the presence of adrenochrome or other quinones, but it is doubtful whether it allows very accurate determination. The same applies to the formation of fluorescence by reduction.

It would appear that any of the methods mentioned will assay single compounds, but that in the assay of mixtures all of them together will have to be considered, combined with physiological methods for unreacted adrenaline and chemical methods for the determination of indol structures. The procedure is tedious and uncertain, since the mixtures undergo further changes while waiting for examination. It is hoped that the results presented will allow in some cases to predict what must and what cannot have happened in the solutions.

(ii) *Interpretation of Physiological Effects of Oxidizing Adrenaline Solutions or Adrenaline Oxidation Products.*—(1) If the effect of a single compound is to be investigated, synthetic oxidation intermediates will have to be chosen; adrenochrome, Lund's fluorescent adrenochrome isomer, the fluorescent reduction product obtained from adrenochrome with BAL, the fluorescent solution obtained by anaerobic alkalization of adrenochrome, and Cohen's oxo-adrenochrome. It is, however, doubtful how long even pure compounds will be stable in contact with metabolizing tissue or enzymes; in the test tube they begin to oxidize after a varying time lag. Probably the best that can be expected is that the reaction mixtures will not contain components of an oxidation level structurally lower than that of the starting material, say that starting from adrenochrome one will not have to count with adrenaline or adrenaline-quinone structures, or, starting from oxo-adrenochrome there will be no leuco-adrenochrome or adrenochrome.

(2) Only at the start of its oxidation will an adrenaline solution contain mainly adrenaline and some adrenochrome. Very soon the solution contains the components of the redox system and their condensation products besides decreasing amounts of adrenaline and increasing amounts of later oxidation products. The same will happen with oxidizing solutions of leuco-adrenochrome. These redox systems are very reactive and the physiological effect of such solutions will be due to individual and possibly specific effects of the components

as well as to the general interference of the redox systems with the metabolism of the tissue or other preparation. Especially delayed effects of an apparently "pure" intermediate, as for example recorded by West (1947) for leuco-adrenochrome, or prolonged tissue reactions must be suspected of being due not to the initial compound but to the formation of complex mixtures or redox systems during the further oxidation.

(3) On alkaline oxidation of dilute adrenaline, temporarily fluorescent, later yellow, but throughout clear solutions are obtained; at no time are noticeable concentrations of red quinones present and no melanin is formed. The same result is obtained at lower pH if excess of hydrogen donors, amino acids, or serum, are present in the reaction mixture; fluorescent solutions are obtained that turn slowly orange or brown, but no extensive melanin formation takes place. It appears to be likely that the oxidation of adrenaline under physiological conditions by non-specific oxidation mechanisms leads also to fluorescent compounds and their decomposition products but not to quinones and melanin, which latter is only formed if the quinones are allowed to accumulate in the solution. The same mechanism may apply to the oxidation of tyrosine, and will result if adrenochrome in moderate amounts is brought into contact with tissue or serum. Therefore the metabolism of adrenaline or any of its early oxidation products in the tissue cannot be accepted as proceeding on the same lines as the atmospheric oxidation in a buffer of the same pH under otherwise equal conditions. Particularly it cannot be accepted that melanin is the end product of the reaction, and the list of possible oxidation intermediates has to be extended beyond the adrenochrome stage. Owing to the high concentration of hydrogen donors, glucose metabolites, amino acids, etc., the quinonoid redox system is present mainly in the reduced form. This fact cannot be without effect on the other oxygen-consuming mechanisms present (cf. the effect of adrenochrome on glucose metabolism (Randall 1946; Wajzer 1946, 1947; Meyerhof and Randall 1948; Marquardt 1947). Regardless of the original composition of the adrenaline oxidation mixture it will very soon contain only insignificant amounts of quinones, and consist mainly of reduced compounds, fluorescent diphenols, and others, and these will determine the physiological effects.

The investigation is being continued in an attempt to localize the peroxidative step in adrenaline oxidation and to differentiate between the fluorescent solutions formed under different conditions.

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VII. REFERENCES

- BACQ, Z. M. (1949).—*J. Pharmacol.* **95** (4): 1.
- BACQ, Z. M., FISCHER, P., and LECOMTE, J. (1948).—*Arch. Int. Physiol.* **56** (1): 25.
- BALL, E. G., and CHEN, T. T. (1933).—*J. Biol. Chem.* **102**: 691.
- BONHOMME, J. (1936).—*Arch. Int. Physiol.* **43** (3): 341.
- BRACONIER, F., LE BIHAN, H., and BEAUDEL, C. (1943).—*Arch. Int. Pharmacodyn.* **69**: 181.
- CHAIX, P., MORIN, G. A., and JEZEQUEL, J. (1950).—*Biochim. Biophys. Acta* **5**: 472.
- COHEN, G. N. (1945).—*Bull. Soc. Chim. Biol. Paris* **28**: 104, 107.
- CONWAY, E. J. (1947).—"Microdiffusion Analysis and Volumetric Error." (Lockwood & Son: London.)
- EVANS, W. C., and RAPER, H. S. (1937).—*Biochem. J.* **31** (2): 2155.
- FALK, J. E. (1949).—*Biochem. J.* **44** (3): 369.
- FISCHER, P., and LECOMTE, J. (1949).—*Arch. Int. Physiol.* **56** (4): 327.
- DA FONSECA-RIBEIRO, D., and CARDOSO, F. A. (1949).—1st Int. Cong. Biochem., Cambridge—Abstracts 102.
- GADDUM, J. H., and SCHILD, H. (1934).—*J. Physiol.* **80**: 9P.
- GREEN, D. E., and RICHTER, D. (1937).—*Biochem. J.* **31** (1): 596.
- HARLEY-MASON, J. (1948).—*Experientia* **4**: 307.
- HARTUNG, W. H. (1946).—*Ann. Rev. Biochem.* **15**: 593.
- HOLTZ, P., and KRONEBERG, G. (1950).—*Biochem. Z.* **320** (4): 335.
- JAMES, W. O., ROBERTS, E. A. H., BEEVERS, H., and DE KOCK, P. C. (1948).—*Biochem. J.* **43** (4): 626.
- KILBY, B. A. (1949).—1st Int. Cong. Biochem., Cambridge—Abstracts 315.
- LOEW, O. (1918).—*Biochem. Z.* **85**: 295.
- LUND, A. (1949).—*Acta Pharmacol. Toxicol.* **5**: 75.
- MARQUARDT, P. (1947).—*Enzymologia* **12** (3): 166.
- MEYERHOF, O., and RANDALL, L. O. (1948).—*Arch. Biochem.* **17**: 171.
- MINZ, B., and PLOTKA, C. (1947).—*C.R. Soc. Biol. Paris* **141**: 108.
- NELSON, J. M., and DAWSON, C. R. (1944).—*Adv. Enzymol.* **4**: 99.
- OBRECHT, M. (1939).—Feldegg A.G., Zurich.
- OPPENHEIMER, C., and STERN, K. G. (1939).—"Biological Oxidation." (W. Junk: The Hague.)
- RANDALL, L. O. (1946).—*J. Biol. Chem.* **165**: 733.
- RICHTER, D., and BLASCHKO, H. (1937).—*J. Chem. Soc.* **1937** (1): 601.
- SCHALES, O. (1938).—*Ber. dtsh. chem. Ges.* **71** (1): 447.
- SHAW, F. H. (1938).—*Biochem. J.* **32** (1): 19.
- SHAW, F. H. (1941).—*Aust. J. Exp. Biol. Med. Sci.* **19**: 151.
- SLATER, E. C. (1949).—*Biochem. J.* **44** (3): 305.
- SUMNER, G. B., and DOUNCE, A. L. (1939).—*J. Biol. Chem.* **127**: 439.
- TORDA, C., and WOLFF, H. G. (1946).—*Arch. Biochem.* **10**: 247.
- TRAUTNER, E. M., and ROBERTS, E. A. H. (1950).—*Aust. J. Sci. Res. B* **3**: 356.
- UTEVSKI, A. M. (1944).—*Adv. Mod. Biol. (U.S.S.R.)* **18**: 145.
- VERLY, W. (1948).—*Arch. Int. Physiol.* **56** (1): 1.
- WAJZER, J. (1946).—*Bull. Soc. Chim. Biol. Paris* **28**: 345.
- WAJZER, J. (1947).—*Bull. Soc. Chim. Biol. Paris* **29**: 237.
- WELCH, A. D. (1934).—*Amer. J. Physiol.* **108**: 360.
- WEST, G. B. (1947).—*Brit. J. Pharm. Chemotherap.* **2**: 121.
- WIESNER, K. (1942).—*Biochem. Z.* **318**: 48.

THE SPERMATOGENIC CYCLE OF THE GUINEA PIG

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Summary

The cell types distinguishable on a nuclear basis during the spermatogenic cycle of the guinea pig are described and an outline of the layer and generation structure of the seminiferous tubule presented.

The resting spermatogonium divides three times, one division product reverting to the resting stage. The relation between changes of cell type in any given layer to the events in other layers of the tubule appears to be rather fixed. Within each layer there is a good synchronization between the individual cells.

On the basis of this correlation, and on data derived from a study of the relative frequency of layers of the various cell types in tubule cross sections, a quantitative formulation of the spermatogenic cycle is made (Figs. 1 and 2). The distribution of phases of the cycle along the tubule is found to be irregular in the guinea pig; the classical spermatogenic "wave" structure is absent. Physiological tubule segments can, however, be delimited. Topographical reconstructions of spermatogonial and spermatocyte divisions do not suggest that there is any simple propagation of a division impulse along the tubule length.

The synchronization within and between layers is interpreted in terms of intercellular and interlayer influences mediated by the Sertoli cells. The attached spermatid categories are considered to be self-limited and are regarded as the tubule pacemakers.

The differences in phase between different physiological tubule segments are considered to be due to the accumulation of small differences in absolute rate of spermatogenesis in these segments during repeated continuous functioning; in discontinuously active testes and pubescent guinea pigs the differences are either not present or very much reduced. The difference in absolute rate of spermatogenesis in tubule segments could arise from differences in blood supply or local differences in the interstitial tissue, or both.

I. INTRODUCTION

The continuously active mammalian testis provides unique adult material suitable for a wide range of biological investigation. It is one of the main sites of cell division in the adult organism and this cell division is controlled temporally, topographically, and quantitatively. A study of these controls in the testis might illuminate the general problems of the initiation and limitation of cell division, both of which are central problems of embryology and oncology.

A further prime problem might also be suitably approached with testicular material; that of differentiation, both histological and cytological. On the one hand an orderly differentiation of cell areas occurs within the seminiferous

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tubule, while within each cell rapid differentiation of a complex nature occurs. These latter changes involve not only a metamorphosis of existing structures but synthesis of entirely new cell organs containing a wide range of chemical components.

The ready availability and uniformity of the material and the presence in any testis cross section of a full range of the stages of the spermatogenic cycle make the material technically very favourable.

The present work, performed in 1947, is purely morphological and largely exploratory. In it an attempt was made to define the "spermatogenic cycle," which has been known for a long time (von Ebner 1871) but which lacked precision in definition. Apart from its intrinsic interest, knowledge of the cycle is required before further questions can be confidently or effectively posed; as in most morphological work of this type, the interpretations are largely speculative and must await experimental procedures for their verification.

II. MATERIALS AND METHODS

Testes from sexually isolated mature male guinea pigs were fixed, after subdivision, in a fluid of the Sanfelice type (2 per cent. chromic acid, 5 parts; acetic acid, 0.5 parts; formalin, 2 parts; water to 10 parts). The paraffin-embedded blocks were cut at 15 μ for study of random cross sections and at 25 μ in the reconstructions.

Staining was by Newton's crystal violet method, usually with light green counterstaining. The plates are from Feulgen light green preparations. In all, testes from some 20 guinea pigs were examined. The stage counts were performed on five unselected animals.

The reconstructions were made on one testis only by the following method. In a microprojector field at a magnification of 83 diameters, a straight tubule was identified and the convoluted seminiferous tubule emptying therein, together with two further marker tubules, was followed and drawn in succeeding sections until it turned. The sheets of drawing paper were accurately superimposed on one another and held together by a clamp. The sheets used for each succeeding drawing were first oriented by reference to the preceding drawing. Successive loops followed in this way were numbered consecutively; each has an up and a down component. As each tubule cross section is drawn it receives its loop number and its up or down designation.

The cytological diagnosis of the tubules in the reconstruction was made initially by reference to every third section. Where necessary every section was examined. In any one section all the relevant numbered tubules in the drawing were diagnosed before proceeding to the next section. In tubule segments cut longitudinally, the direction from the straight tubule was determined and where more than one stage of the cycle was present in this segment, the progression was scored. The evanescent cell types (diplotene and the spermatogonial and spermatocyte divisions) rarely form a complete layer. They are shown in the diagrams as a complete layer because of graphical demands. Secondary spermatocytes were not scored.

The reconstructions of the division complexes were made on more or less straight lengths of tubule by recording section number (to give the longitudinal dimension), the tubule radius (to give the circumferential dimension), and the arc subtended by the cells under study and its radial position in the microscope field.

III. THE CELL TYPES DISTINGUISHED

A general description of the cytological aspects of spermatogenesis in the guinea pig has been given by Moore and Walker (1905), Stevens (1911), Harman and Root (1926), and League (1928).

The cell types distinguished in the present investigation are as follows:

(a) *Spermatogonia*

(i) *The Resting Spermatogonium* is a relatively large cell situated immediately beneath the basement membrane. The number varies from two to five per 15 μ tubule cross section. The nucleus is poorly staining with a fine chromatin network in which one to seven small spherical chromatin nucleoli are present. The cell is not recognizable during the time when the products of its division are passing through the preleptotene phase but reappears when these have passed into early leptotene.

(b) *Spermatocytes*

(ii) *Preleptotene*.—This cell is smaller, the chromatin being more evident and in the form of small irregular lumps. The term as used here does not designate an entirely pure cell type inasmuch as some of the cells may not have completed their division cycle. The designation is given largely on histological criteria and implies the presence beneath the basement membrane of a more or less complete layer of cells not fitting into the other categories recognized.

(iii) *Leptotene*.—Owing to entry into the growth phase this and succeeding cells show increases in volume. Fine chromosome threads are scattered more or less randomly in the nucleus except where they converge on several large, irregular, deeply staining, chromatin nucleoli.

(iv) *Zygotene*.—In the great majority of cases this stage is readily recognized by the chromosome polarization, the centre being away from the basement membrane and oriented towards the centrioles. Owing to fusion the chromosomes are thicker than in leptotene. With the exception of a difficultly visible sex complex the heterochromatic bodies of the leptotene have disappeared.

(v) *Pachytene*.—This stage is delimited from the zygotene by the resolution of the bouquet, the chromosomes now filling the nucleus. There is an increased chromosome diameter and variation in staining intensity along their length, together with some blurring of the edge. The sex complex shows a progressive increase in stainability and size.

(vi) *Diplotene* is recognizable by a relatively sudden contraction of the chromosomes and their more or less radial arrangement. Chiasmata are discerned with difficulty. These cells rarely form a complete layer.

(vii) *Second Spermatocytes* are smaller than the above and the chromatin is in irregular lumps, larger than those visible in the preleptotene cells.

(c) *Unattached Spermatids*

(viii) *Early Solid Spermatid* is the immediate product of the second spermatocyte division. It is distinguished from the latter by its smaller size and the greater regularity of outline of the heterochromatic bodies (2-5), and from the solid spermatid by the greater number and smaller size of the heterochromatic bodies.

(ix) *Solid Spermatid* is so called, as was the above, because of the organization of the chromatin into relatively large, roughly spherical masses (1-2).

(x) *Open Spermatid* is so named because of the disappearance of the heterochromatin present in the solid spermatid. The chromatin becomes more evenly distributed but tends to occur in fine, irregular strands.

(d) *Attached Spermatids*

(xi) *Attached Spermatid* designates a cell at the time of and shortly after its attachment to the Sertoli cell. The nucleus is ovoid instead of spherical as in the open spermatid and the chromatin forms a fine regular reticulum. A pyramidal developing acrosome is frequently visible and the cytoplasm becomes oriented towards the lumen of the tubule.

(xii) *Elongating Spermatid*.—The elongation of the cytoplasm is more marked and flagella are distinguishable. The nucleus is in the form of a thin, ovoid plate and thus usually appears as a deeply staining rod.

(xiii) *Late Spermatid*.—In this cell type there is a marked loss of cytoplasm from the centripetal end; densely staining granules are often visible in this area.

(xiv) *Spermatozoa*.—While still attached to the Sertoli cells these cells are histologically indistinguishable from sperm present in the tubule lumen. The loss of cytoplasm begun in the late spermatid is now complete.

Judging by the ease with which most of these stages are recognized (early solid spermatid and unpolarized zygotene are exceptions) the stages represent critical phases in the life history of the cells and the change from one to another is more or less saltatory. For a number of reasons the terminology used in some of the figures does not correspond with that detailed above. The equivalent terminology is given in the legends to these figures.

IV. THE HISTOLOGICAL ORGANIZATION OF THE TESTIS

In order to clarify the work following, an outline of the histological organization of the testes seems desirable. As in other mammalian testes (e.g. Curtis 1918; Johnson 1934; Huber and Curtis 1913) the guinea pig testis appears to

contain multiple arched tubules entering the rete by a short, straight tubule. In the mature guinea pig seminiferous tubule there are never less than three generations of cells present and for a short period of the cycle four generations occur. Omitting the resting spermatogonia, these are as follows:

(i) *First Generation and Layer*.—Gonial divisions give rise to a fairly complete preleptotene layer. This passes through relatively short leptotene and zygotene and enters the long pachytene stage. At mid pachytene it is pushed into the second layer by the development of new preleptotene layer above it.

(ii) *Second Generation and Layer*.—The primary spermatocytes complete the second half of pachytene, divide, and pass through the solid spermatid stages. Owing to further preleptotene generation arising they then become the third generation and layer.

(iii) *Third Generation and Layer*.—The early open spermatids complete their life history, become attached to the Sertoli cell, where they proceed through the elongating spermatid stage. A further preleptotene layer then relegates them to the fourth layer and generation.

(iv) *Fourth Generation and Layer* is only present for a short time, during which the late spermatid and attached sperm stages are undergone. It ceases to exist when the sperm becomes detached from the Sertoli cell. A quantitative formulation of this layering of the tubule is given in Figure 2.

V. TIME RELATIONS OF THE PRIMARY SPERMATOCYTE

The method used for timing in this section is based on changes occurring in other tubule layers. The validity of the method will be shown in the next section.

TABLE 1
NUMBER OF DIVISIONS UNDERGONE BY A RESTING SPERMATOGONIUM

Testis	Ratio SC/RS	Ratio Z/SC	Spermatogonial Division Products
1	2.45	2.85	7.0
2	2.47	2.90	7.15
3	2.40	2.83	6.8
4	2.50	2.90	7.2

SC = Sertoli cell. RS = Resting spermatogonium. Z = Zygotene.

(a) *Spermatogonial Divisions and Preleptotene*

The preleptotene cells arise by multiple division of the sparsely distributed resting spermatogonia. One of the products of the divisions reverts to the resting stage until the next division cycle commences. The resting spermatogonium is thus visible for about three-quarters of the interdivision period (cf. Fig. 1).

By reference to the ratio between the number of zygotene cells and Sertoli cells and between resting spermatogonia and Sertoli cells (these cells having

almost identical nuclear diameters) it is possible to estimate the number of divisions made by the resting spermatogonium during its cycle of division. The results of such a study are shown in Table 1, 150 Sertoli cells being counted in each case.

It will be seen that each resting spermatogonium gives rise to seven spermatocytes. Since one of the products of division reverts to the resting spermatogonium, the number of products is actually eight, or the cell divides three times. This is considerably less than the number of divisions recently recorded by Roosen-Runge (1950) in the rat.

The relative time relations of the spermatogonial divisions are shown in Table 2, where they are correlated with the other cell types present in the tubule cross section.

TABLE 2
RELATIVE TIME RELATIONS OF THE SPERMATOGENIAL DIVISIONS

Spermato- cyte Category	Unattached Spermatid Category	Attached Spermatid Category	Preleptotene	Testis					Totals	%
				1	2	3	4	5		
Pachytene	Solid	Elongating	—	7	25	8	10	4	54	34
Pachytene	Solid	Late	—			3			3	2
Pachytene	Solid	Late	+		6			2	8	5
Pachytene	Solid	Late	+			2			2	1
Pachytene	Open	Late	—		4	9	8	5	26	16
Pachytene	Open	Late	+	5	2	5	7	9	28	17
Pachytene	Open	Sperm	—	2		2	1		5	3
Pachytene	Open	Sperm	+		2	5	8	8	23	15
Pachytene	Open	—	—	2			1		3	2
Pachytene	Open	—	+			3		1	4	3
Totals				16	39	37	38	30	156	

At first sight the table suggests that the spermatogonial divisions are spread over a wide sector of the cycle. It also suggests that there may be some variation in timing in different individuals: animal 2 shows an earlier initiation of the divisions than the other animals.

On further analysis of the data, however, a narrowing of the spread is evident. Although some 42 per cent. of the total divisions recorded occur in tubules containing the solid type of unattached spermatid, in only 13 per cent. of these cases has the division proceeded far enough to give a recognizable layer of preleptotene cells. After the transformation of the solid to the open spermatid 58 per cent. of the total recorded divisions occur but in this case a preleptotene layer is distinguishable in 60 per cent. of these tubules. The divisions can thus be regarded as being initiated towards the end of the life history of the solid spermatid in the second tubule generation, and continuing for part of the life history of the open spermatid. In the life history of the open spermatid, as judged from the concomitant changes undergone by the

attached spermatid category, it will be seen that the divisions are well advanced during the late stage of the latter category (52 per cent. preleptotene present) and virtually complete by the time of the sperm stage (82 per cent. preleptotene present).

(b) *Leptotene*

The occurrence of the leptotene stages in relation to the stages of cells in other layers of the tubule is shown in Table 3. It will be seen that the change

TABLE 3
LEPTOTENE IN RELATION TO CELLS IN OTHER LAYERS

Spermatocyte Category	Unattached Spermatid Category	Attached Spermatid Category	Testis					Totals	%
			1	2	3	4	5		
Pachytene	Open	Sperm	0	5	1	0	2	8	2
Pachytene	Open	Absent	44	77	79	62	61	320	84
Pachytene	Nil	Attached	14	2	19	2	18	55	14
Diplotene and divisions	Early solid	Attached	0	1	0	0	0	1	0.2
Totals			58	82	100	65	81	387	

from preleptotene to leptotene occurs in rather rigid relation to events in other layers of the tubule. It is initiated at the time of release of the mature sperm from the Sertoli cell and is completed shortly after the next spermatid group becomes attached. In other words, leptotene is contemporaneous with the stage of the free Sertoli cell.

TABLE 4
ZYGOTENE IN RELATION TO CELLS IN OTHER LAYERS

Spermatocyte Category	Unattached Spermatid Category	Attached Spermatid Category	Testis					Totals	%
			1	2	3	4	5		
Pachytene	Open	Nil	0	4	3	1	1	9	2
Pachytene	Nil	Attached	61	49	17	54	41	212	43
Diplotene	Nil	Attached	25	57	45	16	39	182	37
Diplotene	Nil	Elongating	4	1	0	2	10	18	4
Nil	Early solid	Attached	0	9	1	1	1	13	2
Nil	Early solid	Elongating	3	36	11	1	9	60	12
Totals			93	156	77	75	101	494	

(c) *Zygotene*

The relation of events in other layers of the tubule to zygotene is shown in Table 4. It will be seen that the leptotene cells enter zygotene at the time when the third generation spermatids are becoming attached to the Sertoli

cell and when the second generation spermatocytes are approaching the end of pachytene. The stage virtually ends by the time these latter cells have become a coherent layer of early solid spermatids.

(d) *Pachytene*

This is by far the longest phase of the entire cycle. It begins shortly after the second generation spermatocytes have divided and ends by the time the first generation spermatids have completed about one-third of their total time in zygotene. By this time the second generation spermatocytes have progressed through the unattached spermatid stages and have become attached to the Sertoli cells.

(e) *Succeeding Stages in Cycle*

The general outlines of the succeeding stages of the cycle (diplotene → sperm) are evident from the data already presented in this section. The principal correlations are the change from attached spermatid to elongating spermatid, which falls in the middle of the spermatocyte divisions; the change from elongating to late spermatid, which falls in the middle of the spermatogonial divisions; and the loss of mature sperm from the Sertoli cell, which occurs at the same time as the change from preleptotene to leptotene.

TABLE 5
TIME RELATIONS OF THE SECONDARY SPERMATOCYTE

Spermatocyte Category	Unattached Spermatid Category	Attached Spermatid Category	Testis				Total
			1	3	4	5	
Diplotene	Nil	Attached	1	2	5	3	11
Diplotene divisions	Nil	Attached	2	7	10	6	25
Divisions	Nil	Attached	1	2	5	3	11
Divisions	Nil	Elongating	2	8	2	4	16
Diplotene divisions	Nil	Elongating	7	3	1	3	14
Diplotene divisions	Early solid	Attached	0	0	5	0	5
Diplotene divisions	Early solid	Elongating	0	2	2	2	6
Divisions	Early solid	Attached	2	0	9	1	12
Divisions	Early solid	Elongating	8	10	4	8	30
—	Early solid	Elongating	4	2	7	4	17
—	—	Attached	0	1	2	6	9
—	—	Elongating	1	5	3	2	11
Totals			28	42	55	42	167

(f) *The Spermatocyte Divisions*

The analysis of the spermatocyte division complex is made difficult by the short time period which it occupies and the fact that only rarely does any one component of the complex form a complete layer in the tubule. The secondary spermatocyte is the least evanescent cell type of the complex and the relationship of this stage to stages in the same and other layers is shown in Table 5. The whole complex occurs when the first generation spermatocytes are in zygotene.

TABLE 6
FREQUENCY OF OCCURRENCE (%) OF THE VARIOUS CELL TYPES IN TUBULE CROSS SECTIONS

Testis	No. of Tubules	Spgn. Div.	Prelep.	Lep.	Zygo.	Pachy.	Diplo.	Sp. Div.	2nd Spc.	Early Solid	Solid	Open	Attached	Elongating	Late	Sperm
1	322	3.9	16.8	18	22	94	7	9.0	9.2	12.5	26	31.5	25	41	5	6.5
2	480	8.2	21.2	17.7	19.6	85	13	9.4	9.0	11	26	34.5	16	47	5.2	10.2
3	550	6.7	19.6	18.2	18.8	93	6	9.6	8	12.4	29	36.5	19	41	8.2	11.6
4	552	6.8	21.4	12	31.2	78	11	8.5	11.7	21.3	23	34.5	25	38	6.7	9.2
5	552	5.5	21.0	15	25.7	81	9	6.2	8.1	20.4	20	37.0	20	42	10.1	12.5
Mean		6.5	20.3	15.8	23.7	85.3	9.35	8.55	9.15	16.1	25	35.1	20.9	42.2	7.3	10.35

The overlap due to the above factors is evident: in 39 per cent. of tubules the secondary spermatocytes are associated with their precursors (diplotene) and in 41 per cent. with their product (early solid spermatid). The mid point of their distribution is centred exactly on the time when the attached spermatid is changing to the elongating spermatid.

VI. QUANTITATIVE FORMULATION OF THE SPERMATOGENIC CYCLE IN TUBULE CROSS SECTIONS

It is evident from the foregoing section that a rather rigidly controlled cycle exists in the guinea pig testis, and that this should be susceptible of a quantitative formulation.

This formulation is possible by means of a relative time scale based on the percentage occurrence of layers of the various cell types in random samples of tubules, together with an overlap factor for the evanescent cell types. Most of the basis for computation of this latter factor has already been given in the previous section.

The basis of the former factors is given in Table 6, where data derived from study of the cell types in the layers of about 2,500 tubule cross sections in five guinea pigs are shown.

The interpretation of the differences found in different animals is rather difficult. Such differences as the zygotene and pachytene figures in testis 4 are due to difficulties of identification, this animal showing very little polarization of the zygotene chromosomes. Identification difficulties explain the high early solid percentage and the low solid percentage in testes 4 and 5. The variation in the percentage of tubules showing divisions is, however, probably indicative of true differences between animals.

Visual representation of these figures and their integration into a cycle is possible by conversion of the percentages into angles ($100 \text{ per cent.} = 360^\circ$). The data already presented on percentage overlaps provide a useful verification of the quantitative relations between contemporaneous events in different generations of cells in the tubule, and permit a resolution of the difficulties of fitting the spermatocyte division complex into the diagram.

When the data are plotted in angular form, Figure 1 results. In this diagram the stages transected by radii describe the cell types in the various layers of the tubule at that particular stage of the spermatogenic cycle. The diagram is transposed to histological form in Figure 2.

The two figures permit an accurate relative time evaluation of the various stages and an accurate statement of contemporaneous events in the different layers of a tubule cross section. The main lack of precision is in the spermatocyte divisions. Since there is considerable difficulty in recognizing first and second divisions on a histological scale it was assumed that the divisions took an equal time, the total calculated division time being halved to give the time of each division. The interaction between divisions and secondary spermatocyte and between divisions and early solid spermatid allows a reasonably precise evaluation of the time relations involved.

animals. Owing to the great toxicity of colchicine for the guinea pig these attempts were unsuccessful.

VII. SPERMATOGENIC PHASES ALONG THE TUBULE LENGTH

The correlation found between the stages in the layers of tubule cross sections prompted a study of the distribution of stages along the length of the tubule.



Fig. 2.—The spermatogenic cycle related to the seminiferous tubule. For further description see Figure 1. Three generations and layers are always present while for a short time a fourth layer is also present.

For this purpose two reconstructions were made: (1) a complete arch of what is evidently at least a double-arched tubule, and (2) portion of another tubule. The graphical reconstruction of this material is shown in Figures 3

and 4. A complete cytological reconstruction of each is shown in Figures 5 and 6, the framework being determined by the graphical reconstruction. Owing to the distortion of length involved in the graphical reconstruction of tortuous tubules the length dimension shown in these reconstructions has little validity.

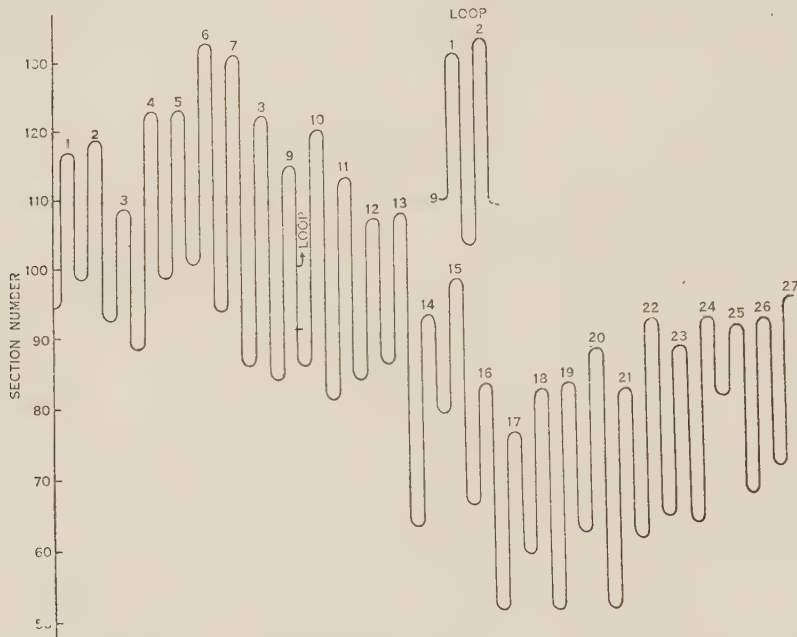


Fig. 3.—Graphical reconstruction of the two tubules studied. Owing to the tortuosity of the tubules the true lengths are considerably greater than those shown in these figures.

In this form the data are rather difficult to interpret. A graphical transposition is shown in Figures 7 and 8, which is more amenable to analysis. The length dimension has been completely eliminated from these figures: they are based entirely on the sequences of stages recognizable in the first layer of the tubule. Owing to the apparent precocity of the spermatogonial divisions in this testis, four generations of cells are present in the tubule for a longer time than found in the testes studied in the previous section. The position of the spermatogonial division is shown by the clear windows in the first generation graph.

The two figures are, in general, similar. The second shows more structure and will be discussed first.

Examination of this second tubule reveals two main features:

(1) As expected from the studies of cross sections, the correlations between layers is good except for the precocity of the spermatogonial division in segments 2, 6, 10, etc. This precocity is compensated for by the open bars in the second generation graph.

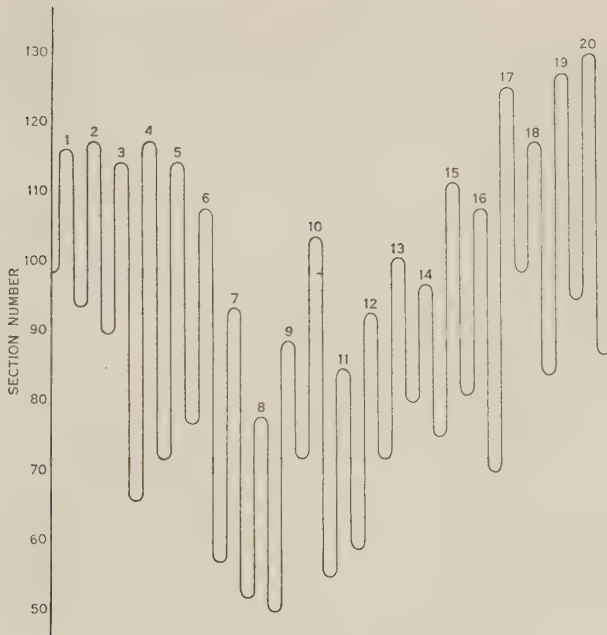


Fig. 4.—As for Figure 3.

(2) No regular cyclic gradient of stages along the tubule length is discernible, as is implicit in text-book discussions of the “spermatogenic wave,” a conception based on the early work of von Ebner (1871, 1888), Benda (1887), and Furst (1887).

With more detailed examination of the figure it is possible to read into it a modified wave concept. The tubule may be divided into a number of autonomous segments in which the concept of propagation of spermatogonial divisions in one or both directions from a point of origin is more or less consistent with the facts. Subdivision of the main segments of the tubule on this basis is shown by the vertical lines beneath the first generation graph where the postulated directions of propagation of the division impulse are shown by the

Fig. 5.—Complete cytological reconstruction of the two tubules, fitted into a four-layer framework. Pachytene has been shown in the second layer throughout: arrows towards the first layer indicate that the pachytene cells are actually in the first layer. The terminology used differs from that defined in Section III. The equivalents are: mature and sperm, middle and late spermatid, early and elongating spermatid, late and attached spermatid. The term very early is used to describe an early stage of elongating spermatid, recognizable by the lesser granule content of the cytoplasm. The “granules” designation in the fourth layer refers to granules present in the centripetal ends of the Sertoli cells shortly after sperm detachment. The numbers above the tubule (with stroke) refer to the loop numbers of Figures 3 and 4, while the numbers 1-44 refer to the segment numbers of Figures 7 and 8. See page 358.

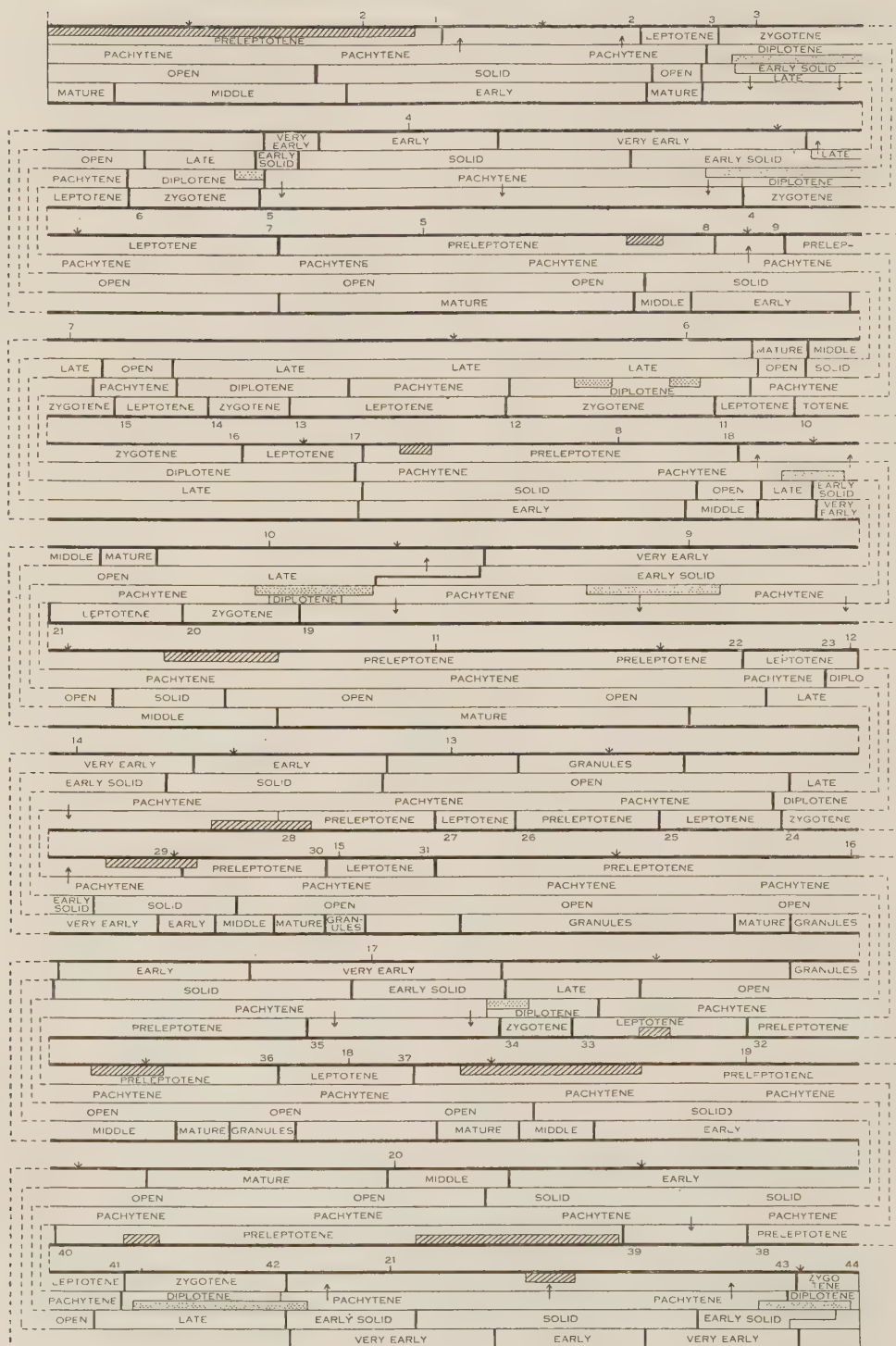
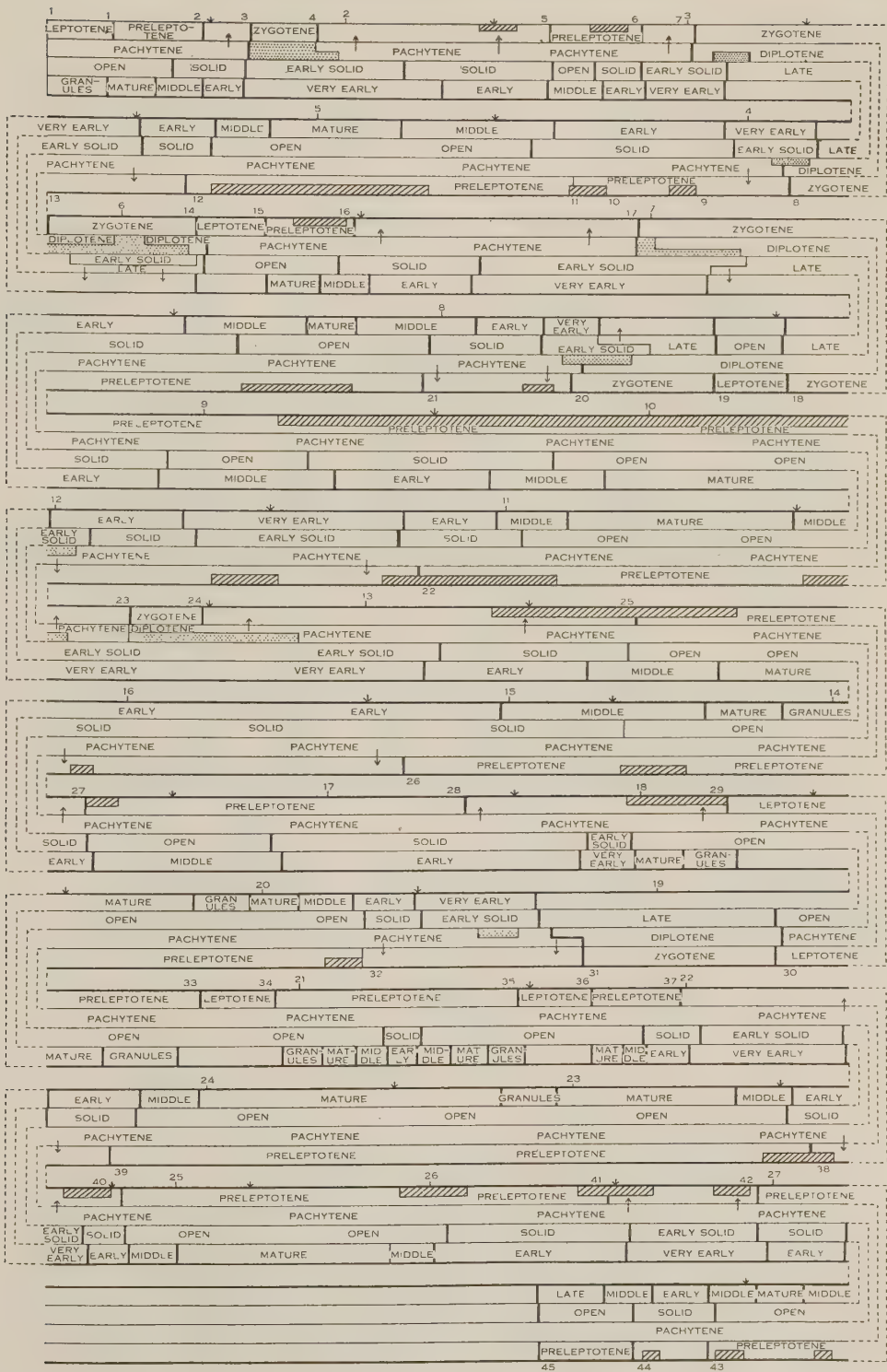


Fig. 5.—Explanation on page 357.



arrows. Even on this limited hypothesis it is difficult to fit some segments into the picture. The difficulty is much greater in the first tubule.

The sequence of stages in the different generations suggests that the hypothetical segments demarcated above are rather constant. This implies that the site of origin of a division wave, if such exists, is rather fixed.

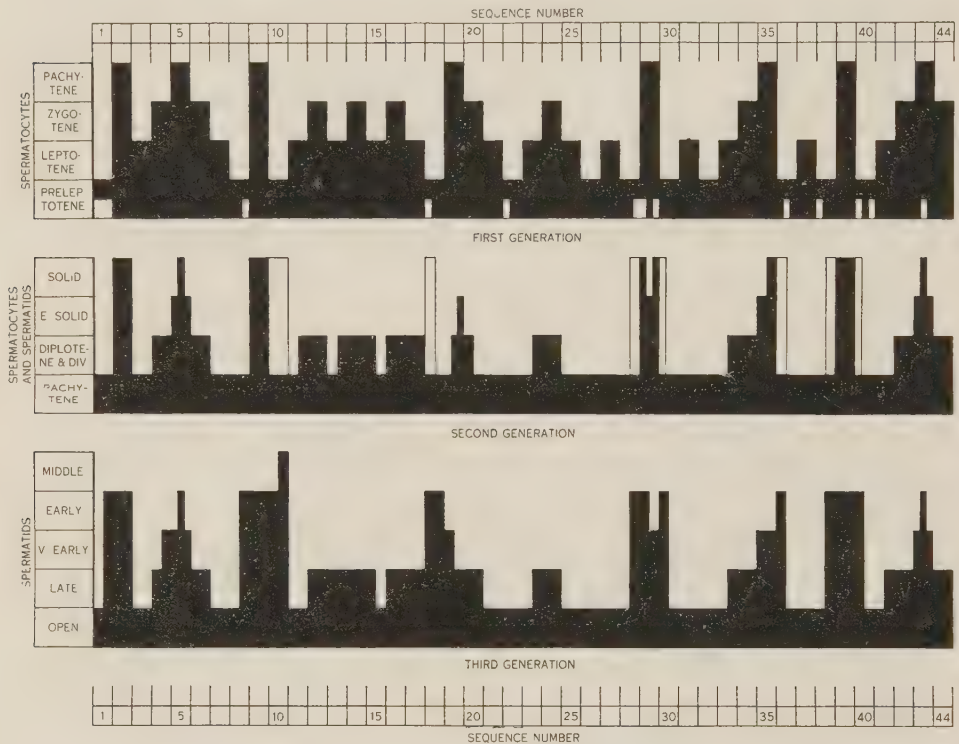


Fig. 7.—Graphical transposition of the data of Figure 5, using the same terminology. Some precocity of the preleptotene generation is apparent in this testis. It is uncertain whether this is due to true precocity of the spermatogonial divisions or to some error of judgment of the preleptotene category. Owing to this fact it has been necessary in parts to show both pachytene and solid spermatid together in the second generation, the solid spermatids appearing as open bars.

VIII. TOPOGRAPHY OF THE SPERMATOGONIAL AND SPERMATOCYTE DIVISIONS

In the previous section it was shown that the sequence of stages along the length of a tubule was consistent with an hypothesis of limited propagation of divisions from a point in one or both directions. In order further to study the possibility of such a propagation, topographic reconstructions of divisions were made by the methods described previously. The topography of typical large-scale spermatogonial and spermatocyte division complexes is shown in Figures 9 and 10.

Considering first the spermatocyte division complex, it will be seen that the observed topography is consistent with a propagation of a division impulse towards the top of the figure. It is, however, evident that complexities are present. There are islands of diplotene present, and the first and the second divisions do not present a uniform wave front: towards the right hand side of the figure the first division has ceased so that second spermatocytes and diplotene are contiguous. The second spermatocyte is the most regular component of the reconstructed complex, probably because it is less evanescent than the other cell types present.



Fig. 8.—As for Figure 7, but transposition of data of Figure 6. Some physiological tubule units are identified by vertical lines below the first generation and the postulated propagation of the spermatogonial divisions in these tubules is indicated by arrows.

Some of this complexity can be resolved by postulating a high ratio between the time required for the divisions and the rate of propagation of the impulse. Variations in competence of cell areas might explain the islands present but some factual knowledge of the existence of determination and the time elapsing between determination and actual division would be required before this concept of competence could be removed from the purely speculative.

These modifying factors apart, it is evident that the situation is more complex than would be expected on the simple wave front theory, although not entirely inconsistent with a limited hypothesis of one-directional propagation of some division-inciting factor.

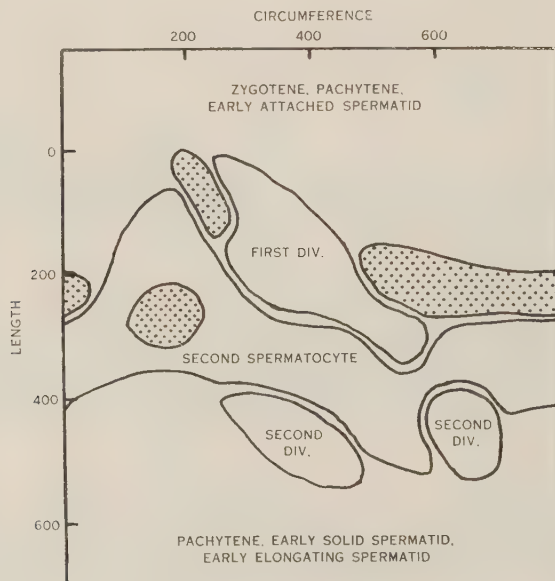
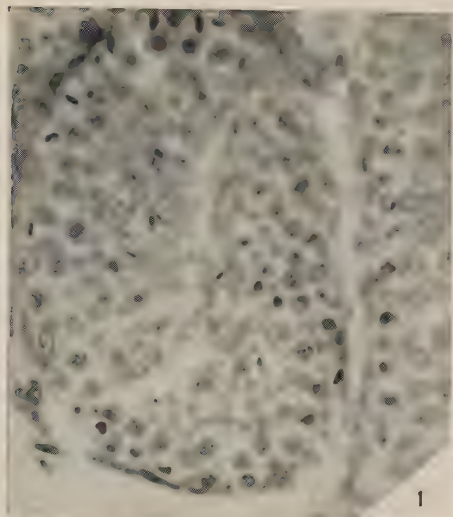


Fig. 9.—Topographical reconstruction of a typical large-scale spermatocyte division complex. The dotted areas contain diplotene cells.

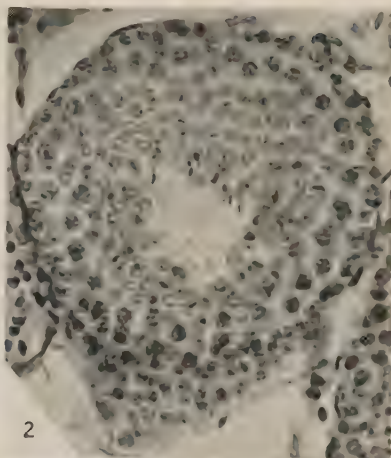
The spermatogonial division reconstruction shows considerably less regularity than found in that of the spermatocyte division. In this case there is little evidence for any propagation of division-inciting factors: over a quite long length of tubule the dividing cells occur in patches with little obvious relation between them. A realization that the divisions seen may represent either first, second, or third spermatogonial divisions does not appear to resolve the difficulty and the conclusion that the spermatogonial divisions do not conform to the wave theory seems warranted. The apparent precocity of the divisions in some testes, and the variations in the percentage of tubules showing spermatogonial divisions (Table 1) are also consistent with this opinion.

On these grounds it would appear that the spermatogonial divisions, while they occur over a restricted part of the cycle (Figs. 1 and 2) are more autonomous than the spermatocyte divisions. Inasmuch as the products of these divisions do not enter meiosis until a definite stage is reached in the third generation, this relative autonomy would not and does not cause any disturbance of synchronization within the tubule.

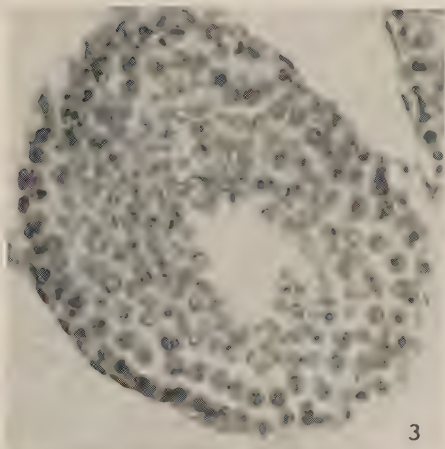
SPERMATOGENIC CYCLE OF THE GUINEA PIG



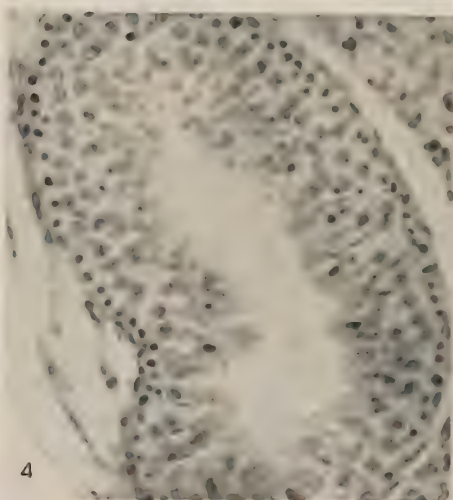
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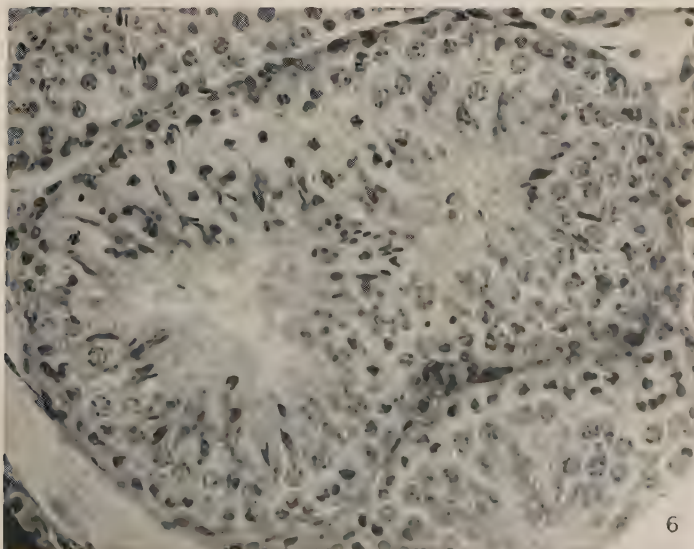
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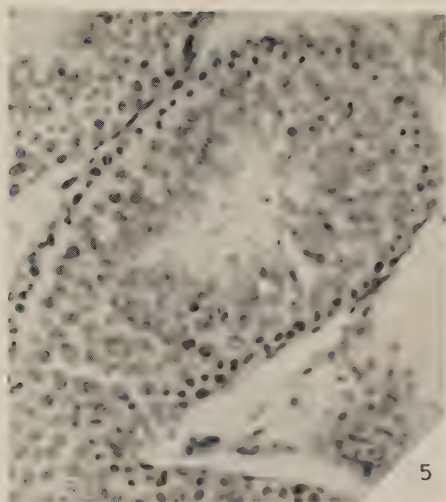
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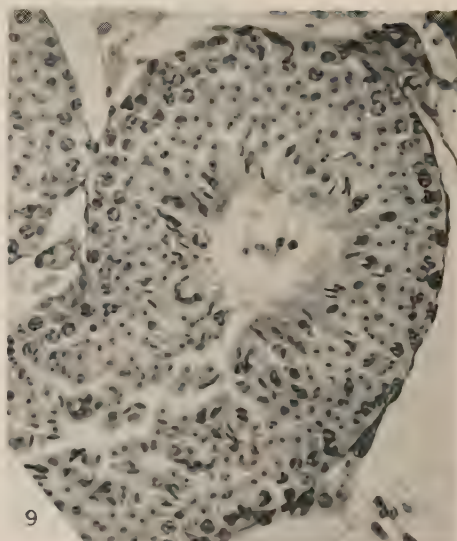
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All figures are from Feulgen light green preparations. A complete key to the illustrations is given in Figures 1 and 2.

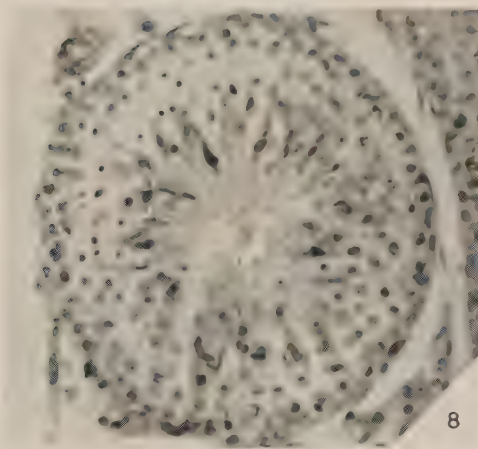
SPERMATOGENIC CYCLE OF THE GUINEA PIG



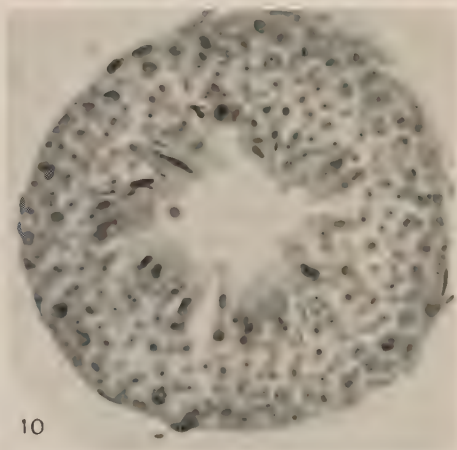
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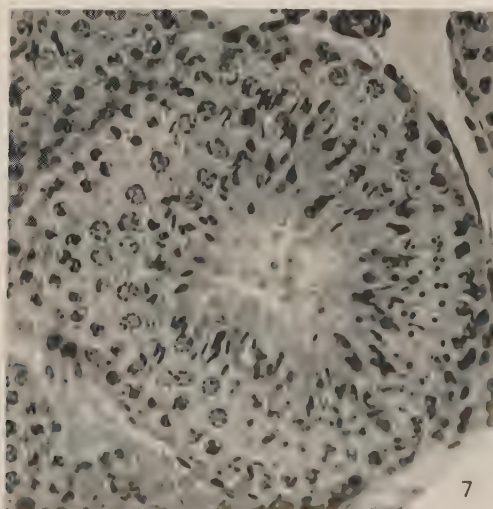
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All figures are from Feulgen light green preparations. A complete key to the illustrations is given in Figures 1 and 2.

IX. DISCUSSION

It has been demonstrated that a fairly rigid correlation of stages exists between different layers of the tubule, while within each layer the cells are well synchronized. Two hypotheses could account for these observations. A rigidly timed self-limitation in the life history of the cells involved in the spermatogenic cycle together with a synchronized entry into the cycle is one possibility. The other is an actual interaction between the layers of the tubule, and between the cells of any given layer.

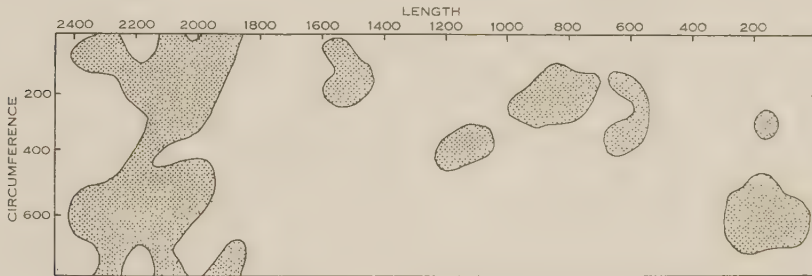


Fig. 10.—Topographical reconstruction of a typical large-scale spermatogonial division complex. There was no preleptotene layer formed in any part of this complex.

There is no direct evidence of the first possibility in the present material, but an assessment may be made from a consideration of other material. The problem is essentially the intercellular variability of self-limited processes. The evidence to be considered is as follows:

(a) The interphase duration of different cells in tissue culture is very variable (Olivo and Delorenzi 1928, 1932). There is less variation in daughter cells but there is no question of the degree of synchronization being comparable to that found in the present material.

(b) In invertebrate ovaries considerable variation among cells appears to exist in the time of reaching a fertilizable condition, a situation that does not depend, as far as can be seen, on different times of entering the growth phase.

(c) In plant anthers there is usually considerable variation in the stages present at any one time. Even adjacent cells may show considerable variation (Smith White, personal communication).

(d) In the cleavage divisions of invertebrate eggs the divisions in different blastomeres are fairly well synchronized in the first few divisions but soon become asynchronous. Even in the early divisions the synchronization may be due to protoplasmic connections between the blastomeres (Gray 1925).

These four cases are found in material in which the cells are discrete and often separated from one another by tissue spaces or thick cell walls. Parallel examples where these barriers are reduced are as follows:

(a) Tissue-culture cells in contact with one another are usually synchronized in division, especially when protoplasmic contact is achieved. Fischer (1946) has considered the organism-like nature of tissue cultures showing these intercellular bridges. Similarly in multinucleate animal cells and syncytia the division of the nuclei is synchronous (Rabinowitz 1941). In *synkarya* in fungi and syncytia of slime moulds, synchronization is also found (Burgess, personal communication).

(b) In invertebrate testes the cells in developing sperm spheres are found to be synchronized, while different sperm spheres, separated by tissue space, show little synchronization. This is the case in insect testes (White 1936) and a similar situation is found in the earthworm (Cleland, unpublished data). This is in marked contrast to invertebrate ovaries, where the developing germ cells are separated from one another.

(c) The most cogent case for intercellular influences leading to synchronization has been presented by Barber (1942) in work on pollen grain divisions. In this work a clear correlation between synchronization and a low cell wall barrier was found. In an earlier paper Barber (1941) has also shown that the hypoploid *Uvularia* pollen grain may survive and divide synchronously with its hyperploid sister cell provided the two are in contact; when separated, the deficient grain dies.

(d) In contrast to the failing synchronization between the blastomeres of developing eggs, it is found that when subdivision of the cytoplasm is prevented the nuclei continue to divide in almost perfect synchrony (Polwzow 1924). The extraordinary synchronization in insect eggs during early development also occurs in a syncytium (Rabinowitz 1941).

The conclusion emerging from the above considerations is that, when cells are not in close connection with one another, biological variability is sufficient to preclude a high degree of synchronization, while when in close contact, especially in protoplasmic contact, synchronization is marked. We may therefore conclude that the high degree of synchronization found in the testis is probably not due to self-limitation of cellular development times but to intercellular and interlayer influence.

The mammalian testis seems histologically well adapted to secure this synchronization: the germinal cells comprising the different layers are embedded in the syncytial Sertoli cell continuum. Since there is no barrier other than single cell membranes in this system, quite long-range synchronization might be predicted; this is found in the testis in any one layer of cells. The concept also permits a credible resolution of the problem of synchronization between layers.

Three phases in the Sertoli cell history are distinguishable: (a) the beginning; and (b) the end of the stage when the Sertoli cell has no attached spermatids; (c) the stage of breakdown of the cytoplasm of the attached spermatids, which is shown by the presence of granules in the degenerating cytoplasm and the centripetal ends of the Sertoli cells. Each of these three stages

corresponds to fundamental changes in different layers. Phase (c) corresponds to the change in the unattached spermatid category from solid to open spermatid. In the former little change in the archiplasmic complex occurs and it may be looked upon as a more or less resting stage as far as morphological work is concerned, while in the latter considerable morphological change occurs. This phase is also associated with the onset of the spermatogonial divisions. The loss of the fully developed sperm from the Sertoli cell (phase (b)) is shortly followed by the entry of preleptotene cells, which are evidently more or less marking time, into the growth phase (leptotene) while the new attachment of spermatids to the Sertoli cell (phase (c)) is associated with the onset of zygotene. The strong polarization of the chromosomes in zygotene and the immense amount of morphological work associated with the attached spermatid stage is followed, after a period, by the onset of the spermatocyte division. It is not improbable that the spermatocyte division onset may be correlated with some unrecognized specific change in the attached spermatid.

If this speculation of interlayer influence is correct we may divide the various stages described into self-limited and correlated types, remembering that within each layer considerable synchronization exists even in self-limited stages because of intercellular influence mediated again by the Sertoli cells. The various stages are thus classified in Table 7.

TABLE 7
SELF-LIMITED AND CORRELATED PHASES IN SPERMATOGENESIS

Self-limited Stages		Correlated Stages		
		Stage	Part Determined	Remarks
Open				
Pacemakers {	Attached spermatid	Gonial div.	Origin	Not very strict
	Elongating spermatid	Preleptotene	End	Strict
	Late spermatid	Leptotene	Origin and end	Strict
	Sperm	Zygotene	Origin	Strict
Early solid spermatid		Pachytene	End	After time lapse or at specific unrecognized stage
Second spermatocyte		Solid	End	Strict
		Open	Origin	Strict
		Spermatocyte div.	Origin	As for pachytene

With this formulation it is evident that if one portion of the tubule is further advanced in the cycle than another, this difference, because of the correlations between layers, will tend to be self-perpetuating. It is also evident that the attached spermatid categories may be regarded as the tubular pace-makers. Being self-limited, variation in the absolute duration of these stages is possible; such variation will automatically induce compensatory changes of duration in the other tubular layers.

Coming now to the sequences of stages along the length of a tubule, the following points have previously been established (Section VII):

(a) Contrary to conclusions of the very early workers, whose examination was more superficial, there is no regular sequence of stages (the so-called "wave") along the length. Curtis (1918), in a study of mouse and rabbit tubules by methods similar to those of the early workers, has also found considerable irregularity in the "waves."

(b) Physiological units of tubule, in which a gradient is present from one end to the other, may be distinguished in at least some parts of the tubule. The ends of these units may be similar or dissimilar in stage to the contiguous unit.

(c) It is unlikely that these gradients are due entirely to any simple propagation of a division-promoting factor along the tubule unit; the contribution of such a propagation to the observed picture could not be excluded, however.

(d) Different units of tubule may vary considerably in length, some being very short, others quite long. Precise values of length are not possible owing to the distortion inherent in the graphical reconstruction but the truth of the above statement is not in doubt.

In attempting to interpret the sequence of stages in a tubule it is instructive to inquire what situation occurs in discontinuously active testes and in the testis of the pubescent guinea pig. In discontinuously active vertebrate testes it appears, either from the figures or from explicit or implicit statements, that spermatogenesis occurs almost simultaneously throughout the testis and does not show the "spermatogenic wave" characteristic of the continuously active testis. This is the case in amphibians (Humphrey 1921; Aron 1926), migratory birds (Bissonnette 1930), and mammals (Courrier 1927; Rasmussen 1917).

In pubescent guinea pigs before the origin of a spermatid layer, or when an early spermatid layer is present in only an occasional tubule, it is found that the difference between tubule cross sections is very much less than is found in the mature testis. As in the latter, there is no difference in the stages reached by contiguous cells in a layer, a fact implying that the intercellular synchronizing mechanisms are fully operative at this time. It would seem therefore that the typical "wave" structure of the seminiferous tubule does not arise except after repeated continuous functioning of the tubule.

The most satisfactory way of accounting for this difference is by the assumption that the absolute times required for a full cycle differ in different parts of the tubule. It will be appreciated that quite small differences in absolute time could, during the sexual lifetime of the animal, lead to the situation found in continuously active testes and would not be discernible in the pubescent animal or in animals maturing only one crop of sperm per season. Calculation makes this fact clear. With a complete cycle taking two weeks, a difference of 1 per cent. in the cycle rate between two segments would in a year cause a 90° shift of phase in Figures 1 and 2. This hypothesis implies

that no fixed relation exists between the stages present in the physiological tubule units delimited in Figure 8, but that the relation will change during the lifetime of the organism and at certain stages the two segments will be in phase.

The postulated differences in cycle rate in different segments might arise in a number of ways: physiological differences in the spermatogonia or Sertoli cells arising during development, local differences in nutrition, or in sex hormone levels.

The first of these factors seems unlikely on general grounds although there is insufficient knowledge of the development of the tubule systems of the testis to provide a firm basis for its rejection. The identity of stages over an interarch junction would also be against the idea, since such a junction would almost certainly imply a fusion of discrete segments of the sex cords.

There is nothing inherently improbable about the second and third factors but there are no real topographic data about either the fine structure of the testicular blood supply or the distribution of the interstitial tissue. The distance between the intertubular blood vessels of the testes and the third generation spermatids which, as indicated previously, may be regarded as the tubule pacemakers, is not inconsiderable, and the possibility that nutrition of these cells may be limiting is good.

Similarly the interstitial tissue hypothesis is attractive. The effect of male sex hormone on the testes has been reviewed by Burrows (1945). The facts are that, by itself, testosterone usually has a depressing effect on spermatogenesis, probably by interference with pituitary function, and that in hypophysectomized animals male hormone has a trophic action for a varying time after operation. A localized action of male hormone on the seminiferous tubule is possible, as indicated by the work of Dvoskin (1943) who has shown that testosterone implants in hypophysectomized rat testes lead to localized maintenance of spermatogenesis. Williams (1950) has recently described interaction between interstitial cells, spermatogenic cells, and Sertoli cells, in transparent chamber grafts in the rabbit.

There is thus no doubt that male sex hormone has some relation to spermatogenesis and it is not improbable that localized differences in interstitial tissue, if they existed, would be capable of giving rise to small differences in cycle rate. Examination of the tubule gradients in testes of animals in which the circulating hormone level has been increased would be interesting in this respect as would those of hypophysectomized animals maintained by male sex hormone. Either the nutritional or the hormonal hypothesis would account for the not infrequent finding of slight phase differences in the two halves of a cross section (e.g. Plate 1, Fig. 2). This formation led Regaud (1900) to postulate a helicoidal division wave.

We have, then, two possible means by which dissimilarity in the spermatogenic rate between different lengths of tubule might arise. In the Sertoli cell mechanism we can account for the self-perpetuating nature of the differences and thus for the fact that this difference in rate is found in all the generations present in the tubule segments. The intercellular synchronization, also

exerted by the Sertoli cells, would ensure that fairly smooth gradients occur within segments and that junctions between segments are not markedly abrupt.

These three factors, together with some small influence exerted by the tendency of self-propagation of divisions, are sufficient to accommodate the four facts, enumerated previously, emerging from the study of the distribution of stages along tubules. They can also account for the differences between discontinuously and continuously active testes; in the former, small localized changes in rate of spermatogenesis are usually not discernible, while in the latter, owing to the cumulation of small differences in successive cycles, these differences are readily visible.

These hypotheses, although accommodating the facts, are still almost entirely speculative. Further work should include:

(a) A study of the changes undergone by Sertoli cells during their cycle of relations to the spermatids. If cyclic changes could be demonstrated the Sertoli cell mediation hypothesis would gain in plausibility;

(b) A study of the effect of sex hormone administration on the cycle as a whole and on the "wave" structure of the tubule;

(c) A closer study of the cytological changes undergone by the attached spermatids in an attempt to link some specific change therein with the induction of the spermatocyte divisions;

(d) A study of the reformation of the "wave" structure in tubules regenerating after a period of artificial cryptorchidism. By such means an estimate of the variation in cycle times of different parts of the tubule should be possible.

X. ACKNOWLEDGMENT

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XI. REFERENCES

- ARON, M. (1926).—Recherches morphologiques et expérimentales sur le déterminisme des caractères sexuels secondaires mâles chez les anoures. *Arch. Biol. Paris* 36: 1.
- ASDELL, S. A., and SALISBURY, G. W. (1941).—The rate at which spermatogenesis occurs in the rabbit. *Anat. Rec.* 80: 145.
- BARBER, H. N. (1941).—Chromosome behaviour in *Uvularia*. *J. Genet.* 42: 245.
- BARBER, H. N. (1942).—The pollen grain division in the Orchidaceae. *J. Genet.* 43: 97.
- BENDA, C. (1887).—Untersuchungen über den Bau des funktionierenden Samenkanälchen einiger Säugethiere, etc. *Arch. mikr. Anat.* 30: 49.
- BISSONNETTE, T. H. (1930).—Studies on the sexual cycle in birds. I. *Amer. J. Anat.* 45: 289.
- BURROWS, H. (1945).—"Biological Actions of Sex Hormones." (Cambridge University Press.)
- COURRIER, R. (1927).—Etude sur le déterminisme des caractères sexuels secondaires chez quelques mammifères à activité périodique. *Arch. Biol. Paris* 37: 175.
- CURTIS, G. M. (1918).—The morphology of the mammalian seminiferous tubule. *Amer. J. Anat.* 24: 339.
- DVOSKIN, S. (1943).—Local maintenance of spermatogenesis in hypophysectomised rats with low dosage of testosterone from intratesticular pellets. *Proc. Soc. Exp. Biol. N.Y.* 54: 111.

- VON EBNER, V. (1871).—Untersuchungen über den Bau der Samenkanälchen und die Entwicklung der Spermatozoiden bei den Säugethieren und beim Menschen. Untersuch. aus dem Inst. f. Phys. Histol. in Göttingen. S.200. (Quoted from Curtis (1918).)
- VON EBNER, V. (1888).—Zur Spermatogenese bei den Säugethieren. *Arch. mikr. Anat.* **31**: 238.
- FISCHER, A. (1946).—"The Biology of Tissue Cells." (Cambridge University Press.)
- FURST, C. (1887).—Über die Entwicklung der Samenkorperschen bei den Beuteltieren. *Arch. mikr. Anat.* **30**: 336.
- GRAY, J. (1925).—The mechanism of cell division. II. *Proc. Camb. Phil. Soc.* **B 1**: 225.
- HARMAN, M. T., and ROOT, F. P. (1926).—Number and behaviour of the chromosomes in *Cavia cobaya*. *Biol. Bull.* **57**: 73.
- HUBER, G. C., and CURTIS, G. M. (1913).—The morphology of the seminiferous tubules of mammalia. *Anat. Rec.* **7**: 207.
- HUMPHREY, R. R. (1921).—The interstitial cells of the urodele testis. *Amer. J. Anat.* **29**: 213.
- JOHNSON, F. P. (1934).—Dissections of human seminiferous tubules. *Anat. Rec.* **59**: 187.
- LEAGUE, B. B. (1928).—The chromosomes of the guinea pig. *J. Morph.* **46**: 131.
- MOORE, J. E. S., and WALKER, C. E. (1905).—The meiotic process in mammalia. Rept. Thompson Yates & Johnston Labs. **7**: 75. (Univ. Liverpool.)
- OLIVO, O. M., and DELORENZI, E. (1928).—*R. Acad. Lincei*. (Quoted from Fischer (1946).)
- OLIVO, O. M., and DELORENZI, E. (1932).—Ricerche sulla velocità di accrescimento delle cellule degli organi. *Arch. exp. Zellforsch.* **13**: 221.
- POLWZOW, W. (1924).—Über die Wirkung der Alkohalnarkose auf die Entwicklung der Seeigeleier. II. *Arch. mikr. Anat.* **103**: 1.
- RABINOWITZ, M. (1941).—Studies on the cytology and early embryology of the egg of *Drosophila*. *J. Morph.* **69**: 1.
- RASMUSSEN, A. T. (1917).—Seasonal changes in the interstitial cells of the testes of the woodchuck. *Amer. J. Anat.* **22**: 475.
- REGAUD, C. (1900).—Direction hélicoïdale du mouvement spermatogénétique dans les tubes séminifères du rat. *C.R. Soc. Biol. Paris* **52**: 1042.
- ROOSEN-RUNCE, E. C. (1950).—Quantitative studies on spermatogenesis in the albino rat. *Anat. Rec.* **106**: 204 (abst.).
- STEVENS, M. M. (1911).—The heterochromosomes of the guinea pig. *Biol. Bull.* **21**: 155.
- WHITE, M. J. D. (1936).—The effects of X-rays on mitosis in the spermatogonial divisions of *Locusta migratoria*. *Proc. Roy. Soc. B* **119**: 61.
- WILLIAMS, R. G. (1950).—Studies of living interstitial cells and pieces of seminiferous tubules in autogenous grafts of testes. *Amer. J. Anat.* **86**: 343.
- WRIGHT, G. P. (1925).—The relative duration of the various phases of mitosis in chick fibroblasts cultivated in vitro. *J.R. Micr. Soc.*: 414.

THE CONVERSION OF CAROTENE TO VITAMIN A IN SHEEP AND CATTLE*

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Summary

It has been established that the conversion of carotene to vitamin A in the sheep occurs in the wall of the intestine. This conclusion is based on surviving tissue experiments in which sections of intestine have been incubated with carotene and the product conclusively identified as vitamin A by colorimetric and spectrophotometric methods. It is further supported by the high vitamin A levels in intestinal as compared with non-intestinal lymph, and similar observations with cattle suggest the intestine as the site of conversion in this species also.

I. INTRODUCTION

The role of the carotenes as precursors of vitamin A was established about 20 years ago, principally by the work of Moore (1929, 1930), and his assumption (Moore 1931) that the liver was the main site of conversion in the animal body has been generally accepted until quite recently. No direct evidence of a satisfactory nature has, however, been advanced in support of this assumption and the observation that the parenteral introduction of carotene into rats failed to relieve symptoms of vitamin A deficiency, the pigment merely accumulating unchanged in the liver, led Sexton, Mehl, and Deuel (1946) to suggest an alternative site of conversion, possibly the wall of the intestine. Subsequent workers (Glover, Goodwin, and Morton 1947, 1948; Mattson, Mehl, and Deuel 1947; Wiese, Mehl, and Deuel 1947; Mattson 1948; Thompson, Ganguly, and Kon 1947; Krause and Pierce 1948), using a number of different techniques, have demonstrated conclusively that in the rat the conversion of carotene to vitamin A occurs in the wall of the intestine.

At this stage other aspects of the carotene metabolism of ruminants were under investigation in this laboratory and, as previous observations had been confined to rats, it seemed of interest, in view of possible species differences, particularly with herbivorous animals, to determine whether the wall of the intestine was also the site of conversion in sheep and cattle. Experiments were undertaken therefore, (1) to attempt, using sections of the small intestine of sheep, a repetition of the surviving tissue experiments by which Wiese, Mehl,

* This investigation forms a section of a thesis submitted in part fulfilment of the requirements for the degree of Ph.D. of the University of New Zealand.

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and Deuel (1947) had so elegantly established the site of conversion in the rat, and, if positive results were obtained, (2) to investigate the mode of transport of the vitamin A from the intestine to the liver, and (3) to determine whether conversion occurred throughout the length of the small intestine or was restricted to certain regions. Since this work was commenced, Thompson, Ganguly, and Kon (1949) have extended previous observations with rats and have shown the site of conversion to be the wall of the intestine in pigs also, while similar findings have been reported for sheep, goats, and rabbits (Goodwin and Gregory 1948), and recently for calves (Stallcup and Herman 1950). Using somewhat different methods, the present investigation has, however, provided further evidence in support of the findings of these later workers and the results are submitted in confirmation of their conclusions regarding the site of conversion in cattle and sheep.

II. EXPERIMENTAL

(a) *In Vitro Conversion Experiments*

Intestines were removed from sheep as rapidly as possible following slaughter and placed in a bath containing Ringer-Locke solution* maintained at about 37°C. Sections (approx. 2-3 ft. in length) were removed from the jejunum and the contents flushed out with Ringer-Locke solution. Colloidal carotene (about 20 ml.), prepared as described by Wiese, Mehl, and Deuel (1947), was introduced into one section from each animal and after ligation at the ends, the tissue was incubated anaerobically in Ringer-Locke solution for 2-3 hours at 37°C. As controls, sections of equivalent length were taken immediately above and below that section into which carotene was introduced. After incubation the colloidal carotene was flushed out with 0.9 per cent. saline. The sections were comminuted in the Waring Blendor under nitrogen with ethanol containing 5 per cent. potassium hydroxide. The suspension was then refluxed until a clear solution was obtained (10 to 15 minutes) and the vitamin A extracted into light petroleum using a method similar to that described by Gallup and Hoeffer (1946) for liver samples. Vitamin A was estimated spectrophotometrically in the light petroleum solutions (concentrated where necessary by evaporation at room temperature under vacuum in a stream of nitrogen) using a Beckman Model D U photoelectric spectrophotometer and applying a three point correction procedure to allow for extraneous absorption (McGillivray 1950). The control sections were assayed in the same way.

The identity of the vitamin A formed in a number of these experiments was confirmed colorimetrically and by a measurement of its absorption spectrum after purification. The extracts containing the crude vitamin A from a number of incubated sections were combined, washed with water to remove traces of ethanol, dried over anhydrous sodium sulphate, and evaporated to

* The Ringer-Locke solution employed had the following percentage composition: sodium chloride 0.9; potassium chloride 0.042; calcium chloride 0.024; sodium bicarbonate 0.05; magnesium chloride 0.02; glucose 0.05.

about 20 ml. under reduced pressure. The absorption curve of this solution was measured and it was then chromatographed on a column of 1:1 magnesium oxide-"Hyflo Super-Cel" as described by Mattson (1948). The chromatogram was developed with light petroleum containing 5 per cent. benzene, and the vitamin A band, detected by its fluorescence under ultraviolet light, separated mechanically and eluted with light petroleum saturated with ethanol. After its absorption curve had been measured the solution was evaporated to dryness and the residue taken up in chloroform. The vitamin A content of this solution was estimated colorimetrically using activated glycerol dichlorohydrin. In addition the absorption curve of the coloured compound formed with glycerol dichlorohydrin was also measured. Glycerol dichlorohydrin was used in preference to antimony trichloride solution since the transitory nature of the colour produced by the latter reagent renders measurement of its absorption curve difficult in spectrophotometers of the Beckman type.

(b) Transport of Vitamin A from the Intestines to the Liver

If conversion of carotene to vitamin A occurs in the wall of the intestine, the vitamin A formed must be transported from there to the liver by either the portal or lymphatic routes or both, and carotene will not appear in the blood or lymph unless the rate of absorption exceeds the rate of conversion to the vitamin. This excess carotene may be transformed to vitamin A at a secondary site of conversion or it may be treated merely as a waste product and decomposed in various tissues. It was apparent therefore that a comparison of the carotene and vitamin A levels in portal and systemic blood and in intestinal and non-intestinal lymph would supply information regarding the site of conversion and the mode of transport of the vitamin or provitamin from the intestine.

Intestinal and non-intestinal lymph glands were removed from a number of sheep immediately following slaughter. Three groups of similar pasture-fed animals were used and sufficient were included in each group to provide about 5 ml. of both types of lymph. In each group the intestinal glands, which included duodenal, jejunal, and ileal, were combined and as much lymph as possible collected from them. In the same way, samples of non-intestinal lymph were collected from various other glands, mainly submaxillary and pharyngeal, from the same animals. Preliminary estimations of carotene and vitamin A were carried out using the method described by Kimble (1939) for blood plasma. Poor recoveries of vitamin A added as internal standard indicated the presence of colour inhibitors in amounts greater than encountered in blood plasma, and for the lymph samples the Kimble method was modified to include saponification as described by Parrish, Wise, and Hughes (1948).

Similarly, samples of intestinal and non-intestinal lymph were obtained from a pasture-fed bullock immediately following slaughter. These were assayed for vitamin A using the modified Kimble method. Sufficient lymph was obtained to carry out the assays in duplicate.

Attempts were made to compare the carotene and vitamin A levels in portal and systemic blood using an anaesthetized sheep following injection into the intestine of an excess of readily absorbable carotene. Although, in a preliminary experiment, the vitamin A level in the portal blood was found to be markedly higher than in the systemic blood, it is doubtful whether reliance can be placed on this observation, since, as pointed out by Goodwin and Gregory (1948), absorption processes in general are retarded by anaesthesia and trauma, and these experiments were not proceeded with.

(c) Region of Conversion

In an attempt to determine whether the conversion could occur at any point along the intestine or was limited to a particular region, whole intestines were incubated with colloidal carotene, using a method similar to that already described for the short sections. After incubation the intestines were cut into short sections, which were assayed separately for vitamin A. These experiments gave variable results owing possibly to injury to portions of the tissue caused during handling. It was possible, however, to obtain an indication of where carotene absorption and conversion occurred by estimating vitamin A in the various intestinal lymph glands. Duodenal, jejunal, and ileal lymph glands were removed from four pasture-fed sheep immediately following slaughter. Insufficient lymph could be obtained from each gland for assay so the assumption was made that the ratio of lymph to gland tissue was relatively constant, and whole glands, after grinding with sand, were assayed separately for vitamin A, using the method already described for the sections of intestine. For plasma and lymph samples it was found that, provided the extracts had been saponified, the three point correction procedure gave results agreeing to within about ± 8 per cent. with the Carr-Price figures.

III. RESULTS AND DISCUSSION

For the surviving tissue experiments it was not possible to deplete the sheep of vitamin A before slaughter or even to maintain them for a short time on a carotene-free diet. In most cases therefore, carotene absorption was proceeding at the time of slaughter and the sections contained appreciable quantities of carotene and vitamin A before incubation. A large number of experiments were carried out on sections of this type and, omitting preliminary experiments, the incubated tissues showed statistically significant increases in vitamin A content of up to 15 per cent. Although this was considered evidence of conversion in the wall of the intestine, the increases were small and the results less convincing than those reported by Wiese, Mehl, and Deuel (1947) for vitamin A-depleted rats. It was possible, however, to carry out a few experiments on intestines from sheep that had been fed a poor quality hay of low carotene content for some time prior to slaughter. The vitamin A content of control and incubated sections from six of these animals is shown in Table 1. Analysis of variance carried out on these figures showed the increase on incubation with colloidal carotene to be highly significant.

The absorption curve for the combined light petroleum extracts from these six incubation experiments is shown in Figure 1, curve 1. Curve 2 gives the absorption of the purified solution obtained after chromatography. This curve resembles that of vitamin A, and application of the three point correction procedure gave an apparent total vitamin A content of 88 μ g. (78 per cent. recovery) as illustrated by curve 3. Curves 4 and 5 represent the impurity apparently present in the original and chromatographed solutions respectively. The total vitamin A content estimated colorimetrically with glycerol dichlorohydrin was 98 μ g. (86 per cent. recovery) and the absorption curve of the glycerol dichlorohydrin addition product agreed closely with that reported for pure vitamin A (Sobel and Werbin 1946).

TABLE 1
VITAMIN A IN SECTIONS OF THE INTESTINAL WALL OF SHEEP AFTER INCUBATION
WITH CAROTENE AT 37°C.

Animal	Vitamin A (μ g.)		
	Control	Incubated	Increase on Incubation
1	8.2	25.2	17.0
2	8.4	20.2	11.8
3	4.8	8.5	3.7
4	9.8	27.3	17.5
5	6.9	19.3	12.4
6	5.2	12.3	7.1
Mean	7.2	18.8	11.6

The vitamin A levels in the intestinal lymph samples collected from the three groups of sheep were 118, 84, and 102 μ g./100 ml. while the corresponding non-intestinal lymph samples contained respectively, 38, 35, and 34 μ g./100 ml. As might be expected from its virtual absence from the blood plasma of sheep, no carotene could be detected in any of the lymph samples. From these results it may be concluded that in sheep the wall of the intestine is a site of conversion of carotene to vitamin A. Eden and Sellers (1948) found the lymphatic route to be the main one by which the vitamin A absorbed was transported to the liver following oral administration to bullocks of emulsified halibut liver oil. From the vitamin A levels found in the intestinal and non-intestinal lymph of sheep in the present investigation, it is apparent that part at least of the vitamin A derived from the conversion of carotene in the wall of the intestine is also transported via the lymphatic route. Similar conclusions, that vitamin A is formed in the intestine and transported partly via the lymphatic system, may be drawn for the pasture-fed bullock. These intestinal lymph samples, taken from two positions along the small intestine, contained 142 and 159 μ g. vitamin A per 100 ml. and two non-intestinal samples 48 and 60 μ g. per 100 ml. Carotene was also present in the intestinal lymph, the two samples assayed averaging 345 μ g. per 100 ml.

The vitamin A levels in the individual intestinal lymph glands of the four pasture-fed sheep are shown in Figure 2. Since only relative figures were required, the average vitamin A content of the glands from each sheep, in $\mu\text{g./g.}$, was calculated and a comparison between sheep made by expressing the vitamin A concentration ($\mu\text{g./g.}$) in each gland as a percentage of the mean for the animal. These percentages are plotted against the relative position of the gland along the intestine also expressed as a percentage of the total length.

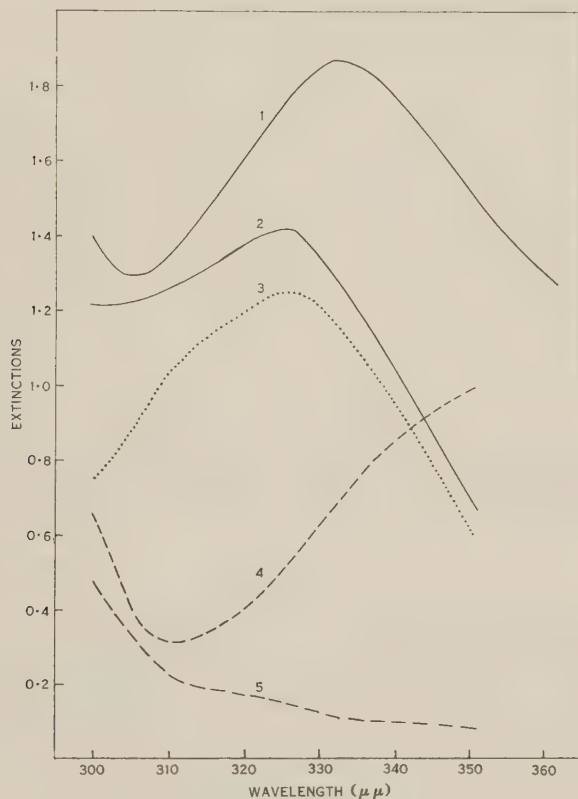


Fig. 1.—Vitamin A formed on incubating intestines at 37°C. with colloidal carotene.

Owing to the variations in size and number of the glands their relative positions are plotted as lines, the lengths of which represent the relative sizes of the glands and give an indication of the length of intestine from which lymph is drained by each gland. The low level of vitamin A and the small size of a number of the glands limits the reliance that can be placed on individual assays but the results obtained from the four sheep indicate that, although there is some carotene absorption and conversion over the whole intestine, maximum vitamin A formation occurs in the upper and lower portions.

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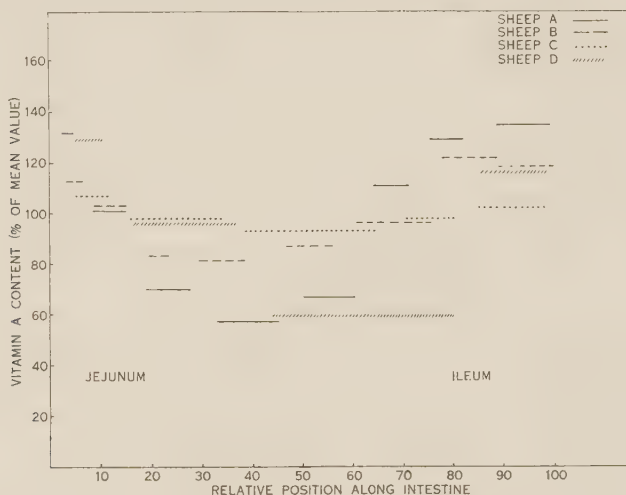


Fig. 2.—Relative vitamin A levels in intestinal lymph glands. Relative position along intestine expressed as percentage of total length.

V. REFERENCES

- EDEN, E., and SELLERS, K. C. (1948).—*Biochem. J.* **42**: xlix.
 GALLUP, W. D., and HOFFER, J. A. (1946).—*Industr. Engng. Chem.* (Anal. Ed.) **18**: 288.
 GLOVER, J., GOODWIN, T. W., and MORTON, R. A. (1947).—*Biochem. J.* **41**: xlv.
 GLOVER, J., GOODWIN, T. W., and MORTON, R. A. (1948).—*Biochem. J.* **43**: 512.
 GOODWIN, T. W., and GREGORY, R. A. (1948).—*Biochem. J.* **43**: 505.
 KIMBLE, M. S. (1939).—*J. Lab. Clin. Med.* **24**: 1055.
 KRAUSE, R. F., and PIERCE, H. B. (1948).—*Arch. Biochem.* **19**: 145.
 MCGILLIVRAY, W. A. (1950).—*Anal. Chem.* **22**: 494.
 MATTSON, F. H. (1948).—*J. Biol. Chem.* **176**: 1467.
 MATTSON, F. H., MEHL, J. W., and DEUEL, H. J. (1947).—*Arch. Biochem.* **15**: 65.
 MOORE, T. (1929).—*Lancet* (i) 499 and (ii) 380.
 MOORE, T. (1930).—*Biochem. J.* **24**: 692.
 MOORE, T. (1931).—*Biochem. J.* **25**: 275.
 PARRISH, D. B., WISE, G. H., and HUGHES, J. S. (1948).—*Anal. Chem.* **20**: 230.
 SEXTON, E. L., MEHL, J. W., and DEUEL, H. J. (1946).—*J. Nutr.* **31**: 299.
 SOBEL, A. E., and WEBBIN, H. (1946).—*Industr. Engng. Chem.* (Anal. Ed.) **18**: 570.
 STALLCUP, O. T., and HERMAN, H. A. (1950).—*J. Dairy Sci.* **33**: 237.
 THOMPSON, S. Y., GANGULY, J., and KON, S. K. (1947).—*Brit. J. Nutr.* **1**: v.
 THOMPSON, S. Y., GANGULY, J., and KON, S. K. (1949).—*Brit. J. Nutr.* **3**: 50.
 WIESE, C. E., MEHL, J. W., and DEUEL, H. J. (1947).—*Arch. Biochem.* **15**: 75.

RUMINAL FLORA STUDIES IN THE SHEEP

III. THE INFLUENCE OF DIFFERENT SOURCES OF NITROGEN UPON NITROGEN RETENTION AND UPON THE TOTAL NUMBER OF FREE MICROORGANISMS IN THE RUMEN

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Summary

The data are presented from a replicated feeding trial designed to determine the influence of different sources of nitrogen, fed at a constant level, upon the nitrogen balances and numbers and types of ruminal microorganisms in growing lambs. Six Merino lambs were fed a basal ration of oaten chaff and wheaten grain supplemented with six sources of nitrogen: linseed meal, subterranean clover seed, whole powdered egg, casein, urea, and urea plus methionine. In each diet the test nitrogen contributed 40 per cent. of the total nitrogen and the crude protein ($N \times 6.25$) content of the whole diet was very close to 10 per cent. All diets were very similar in crude fibre and gross energy content. Additional data for certain of these diets were obtained with seven mature Merino wethers.

The mean biological values of the nitrogen of the different rations as fed to the lambs were: linseed 79.7 ± 2.59 ; subterranean clover seed 83.0 ± 3.43 ; egg 86.7 ± 4.28 ; casein 82.0 ± 6.05 ; urea 68.6 ± 1.52 ; urea plus methionine 75.2 ± 1.48 . The biological value of the nitrogen of the whole powdered egg ration was significantly greater ($P < 0.01$) than that of linseed, of linseed significantly greater ($P < 0.01$) than that of urea plus methionine, and this significantly greater ($P < 0.01$) than that of urea. The values for the casein and subterranean clover seed rations were significantly greater than that of the urea ($P < 0.01$) and urea plus methionine ($P < 0.05$) rations but were not significantly different from each other nor from the other protein nitrogen sources.

The mean concentrations of ruminal bacteria on the different rations were found to be: linseed 23.9 ± 8.46 ; subterranean clover seed 25.2 ± 10.5 ; egg 41.2 ± 5.23 ; casein 42.8 ± 9.94 ; urea 17.7 ± 2.09 ; urea plus methionine 43.7 ± 9.12 million per cu. mm. The ruminal bacterial numbers were highly significantly greater ($P < 0.01$) for the egg, casein, and urea plus methionine diets than for the linseed, subterranean clover seed, and urea diets.

It is concluded that:

(i) Different sources of nitrogen can vary markedly in their biological value, i.e. capacity to promote nitrogen retention in growing lambs.

(ii) Different sources of nitrogen can vary markedly in their capacity to promote bacterial growth in the rumen of both growing lambs and mature sheep.

(iii) The value of methionine, as a supplement to urea, in improving nitrogen retention in growing lambs is due largely to its stimulating effect on bacterial growth in the rumen, thus increasing the amount of bacterial protein available to the host.

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The numbers of ruminal protozoa were found to be highly variable and to bear no obvious relationship to the diets fed.

Some of the morphological characteristics of the ruminal bacteria on the various diets are presented and discussed.

I. INTRODUCTION

In an experiment designed to determine the effects of varying intakes of protein, from a restricted source, upon the numbers of "free" ruminal micro-organisms in the sheep Moir and Williams (1950) found an extremely high correlation between the levels of protein intake and the total numbers of free micro-organisms in the rumen. They concluded that, under the conditions of the experiment, the number of organisms was determined by the protein intake and that a relatively constant proportion of the dietary protein (about 50 per cent.) was converted to bacterial protein. It was realized that these results might apply to only one type of ration and to one source of protein (casein) supplementing a basal diet of oaten hay and starch. No data are available, so far as is known, on the effect of different sources of nitrogen, fed at a constant level, upon ruminal flora numbers, although there is considerable information from American sources on the utilization by the sheep of different nitrogen sources.

Johnson *et al.* (1942, 1944) presented evidence indicating that, up to a level of 10-12 per cent. crude protein ($N \times 6.25$), a considerable proportion of the protein ultimately utilized by the ruminant is microbial protein regardless of the nature of the dietary nitrogen. They comment on the frequency with which biological values close to 60 have been obtained with ruminants fed a wide variety of rations in which the protein level is about 10-12 per cent. Exceptions to this generalization occurred, however, in the work of Sotola (1930) and Turk, Morrison, and Maynard (1934, 1935). Loosli and Harris (1945) obtained a marked improvement in the nitrogen balance of lambs when methionine was added to a urea ration. In a more extensive study Lofgreen, Loosli, and Maynard (1947) confirmed this result with respect to urea and urea plus methionine, and also found significant differences in the biological values of various nitrogen sources for the growth of lambs. A biological value of 80 was found for whole egg protein, 76 for linseed meal protein, 74 for urea plus methionine, and 71 for urea. It should be noted that all these values are appreciably higher than 60, the figure quoted by Johnson *et al.* (1942, 1944), although the nitrogen intake in each case was equivalent to 10 per cent. crude protein ($N \times 6.25$).

The work reported in this paper constitutes a repetition of the work of Lofgreen, Loosli, and Maynard (1947), although a wider range of nitrogen sources is employed, but it goes much further, since the influence of the rations on the microflora and fauna of the rumen is also assessed.

II. EXPERIMENTAL

(a) Design of Experiment

The original feeding trial was designed on the basis of a 6×6 latin square in which six lambs, six trials, and six rations with different nitrogen sources were used. Unfortunately, this design could not be adhered to because of the poor consumption of certain of the diets by some of the lambs. The number of trials was therefore extended to obtain a sufficient number of satisfactory determinations. The significance of the results was tested by the use of Fisher's "*t*" test, no analysis of variance being possible.

In addition some further trials were carried out with seven mature wethers because of the great variability shown by the lambs on certain of the diets with respect to numbers of ruminal microorganisms. The mature wethers are distinguished from the lambs by the letter X following the number of the animal in the tables.

(b) Experimental Animals

Six Merino wether lambs were selected from a larger group of similar breeding for evenness as to age and appearance. They were all between 5 and 7 months of age and 60 and 70 lb. live weight. The seven mature Merino wethers were also selected from a larger group for evenness of size and appearance.

(c) Rations

The following six sources of supplementary nitrogen were used: linseed meal, subterranean clover seed, whole dried egg, casein (acid-precipitated), urea, and urea plus methionine. In each of the rations fed, approximately 40 per cent. of the total nitrogen of the ration came from one of these sources. The rest of each ration was made up of oaten chaff (40 per cent.), wheaten grain (33.3 per cent.), a mineral supplement, a small amount of molasses, and varying amounts of starch. The starch was varied in order that the diets should be of similar gross energy value, dry matter, nitrogen, and crude fibre contents. The crude protein ($N \times 6.25$) content of all the rations was very close to 10 per cent. The compositions of these rations are given in Table 1.

For all rations except the egg, the constituents other than chaff were thoroughly mixed and bound together in the form of "nuts" with a small amount of watered molasses, the resultant mix being dried for 48 hours at 55°C . The powdered egg was kept separate in air-tight tins in a refrigerator and the required weight added to the rations each day. The rest of the constituents of the egg ration were treated in the same way as the other rations.

The diets were weighed into paper bags as required, a known weight of the mixture being added to the required weight of chaff. In each case the ratio of chaff to mixture was 2 : 3. Samples were taken of each mix and of each bag of chaff for analysis.

The actual daily consumption by each animal, i.e. after making allowance for food residues, is given in Appendix I.

(d) Treatment of Animals

Each feeding period extended over 24 days, the last 10 days of which constituted the collection period. There were no rest periods between treatments. Previous experience (Moir and Williams 1950) has shown that a 14-day pre-collection period is normally sufficient to allow for adjustments of numbers of microorganisms to dietary changes where sheep have been fed for some months on dry feed. Certain types of organisms, however, persist for much longer periods in some cases, as will be described later.

TABLE 1
PERCENTAGE CONSTITUENTS IN DIET

Diet	C Egg	E Urea	F Urea and Methionine	B Sub. Clover Seed	D Casein	A Linseed
Chaff	40	40	40	40	40	40
Wheat	33.33	33.33	33.33	33.33	33.33	33.33
Egg	9					
Urea		1.44	1.43			
DL-Methionine			0.2			
Sub. clover				12.62		
Casein					5.13	
Linseed						11.75
Starch	8.42	16.0	15.78	4.8	12.28	5.67
Molasses	6.67	6.67	6.67	6.67	6.67	6.67
NaCl	0.58	0.58	0.58	0.58	0.58	0.58
Dicalcic phosphate	1.0	1.0	1.0	1.0	1.0	1.0
CaCO ₃	1.0	1.0	1.0	1.0	1.0	1.0
Total	100.0	100.0	100.0	100.0	100.0	100.0
Crude protein (%)	9.95	9.92	10.03	9.67	9.67	9.62

The sheep were maintained in metabolism crates throughout and were fed the whole of their daily ration at 9 a.m. each day. During the 10-day collection period urine, faeces, and any feed residues were collected daily immediately prior to feeding. On the seventh and ninth days of this period, samples of rumen contents were withdrawn by stomach-tube (Moir and Williams 1950) at 3 p.m., i.e. six hours after feeding. The sheep were weighed on the first, thirteenth, and twenty-fourth days of each treatment. The average of the last two weights was used in the calculations of the biological values of the nitrogen of the diets. Adequate tap water was before the sheep at all times.

(e) Treatment of Urine, Faeces, and Feed Residues

The daily collections of urine, faeces, and feed residues were treated in the manner described by Moir and Williams (1950).

(f) Counting Techniques

The counts for the total concentrations of free ruminal bacteria and of protozoa were made as described by Moir (1951) following the method of Gall,

Stark, and Loosli (1947). Carbol-fuchsin smears were used to study the morphology of the bacteria. A qualitative estimate of the proportion of different types of organism was made by six separate counts of the numbers of each type in a defined field over the smeared area. Gram stains were also made for one sample from each treatment, from which an estimate of the proportion of Gram-negative to Gram-positive organisms was made.

Some comment on the accuracy of the counts for total free bacteria and of the extent of diurnal and day-to-day variations in individual animals on a fixed dietary régime is appropriate at this point, since the validity of any dietary treatment differences is obviously influenced by these factors.

The satisfactory relationship that exists between stomach-tube samples and the free microorganisms within the rumen has been discussed by Moir and Williams (1950) and need not be mentioned again. The relationship between the numbers of organisms counted in a nigrosine smear and the numbers actually present in the sample, however, is not nearly so satisfactory. It is exceedingly difficult to distinguish with certainty between artifacts and bacteria when their size is less than about $0.5\ \mu$. The counts presented in this study arbitrarily exclude all bacteria less than about $0.5\ \mu$, even though the presence of more minute organisms can be demonstrated in stained preparations. As a result, the numbers of organisms counted are slightly underestimated. Nevertheless, repeated checks by different workers using this technique, both in this laboratory and elsewhere, have given very similar results on the same samples and it is not unreasonable to argue that they are valid for comparative purposes, i.e. for comparing the effects of different dietary treatments. The value of phase contrast microscopy in overcoming this difficulty is being investigated at the present time.

Day-to-day variations in counts made on samples taken from the same sheep at the same time in relation to feeding are, in the experience of this laboratory, usually small. Moreover, the counts presented in this paper are the average of two counts made on *each* of two samples, one taken on the seventh and one on the ninth days of the collection period. Diurnal variations are considerable and are the subject of a separate study to be reported later, but it can be stated that for most types of rations there is a diurnal pattern in which the concentration of ruminal bacteria is at a minimum in the early morning before feeding and is maintained at a significantly higher level for a period of about seven hours, i.e. from three to ten hours after feeding. The sampling time used in this investigation, namely six hours after feeding, represents the time at which the concentration of ruminal bacteria is, in our experience, most likely to be near its maximum.

III. RESULTS

The complete nitrogen balance data for each lamb for each experimental period are presented in Appendix I. In Appendix II the individual ruminal bacteria and protozoa counts are presented for both the lambs and the mature wethers included in the later stages of the experiment. The mean values for each treatment, together with their standard errors, are given in Table 2.

The biological values of the nitrogen of the rations were calculated from the nitrogen balance data by using the figures for metabolic faecal nitrogen (5.55 mg. N per 100 g. dry matter intake) and for endogenous urinary nitrogen (0.035 g. N per kg. body weight) given for lambs by Harris and Mitchell (1941).

TABLE 2
AVERAGE RESULTS FOR EACH TREATMENT

Diets	A	B	C	D	E	F
	Linseed	Subterranean Clover	Egg	Casein	Urea	Urea and Methionine
Calc. biological value (%)	79.7 ± 2.59	83.0 ± 3.43	86.7 ± 4.28	82.0 ± 6.05	68.6 ± 1.52	75.2 ± 1.48
"True" digestibility of nitrogen (%)	89.5 ± 4.71	86.6 ± 3.43	94.2 ± 5.23	90.8 ± 2.92	92.5 ± 2.93	92.2 ± 2.16
Digestibility of dry matter (%)	73.1 ± 2.21	68.5 ± 3.76	73.9 ± 4.35	74.5 ± 3.08	74.6 ± 2.19	73.8 ± 2.21
Rumen bacteria (millions per cu. mm.)	23.9 ± 8.46	25.2 ± 10.5	41.2 ± 5.23	42.8 ± 9.94	17.7 ± 3.09	43.7 ± 9.12
Methionine (g.) (esti- mated) per 650 g. dry matter ration	1.6	1.0	2.1	2.0	0.9	2.3

Inspection of the results of Table 2 suggests that there are real differences in the biological values of the various nitrogen sources. Statistical analysis of these data reveals the fact that certain of these differences are highly significant. Thus the mean biological value of the egg protein is significantly ($P < 0.01$) higher than that of the linseed meal protein, the linseed meal protein significantly ($P < 0.01$) higher than the urea plus methionine, and the urea plus methionine significantly ($P < 0.001$) higher than the urea. The biological values of the casein and the subterranean clover seed diets were not significantly different from each other or from the egg and linseed meal diets but they were both significantly higher than the urea plus methionine ($P < 0.05$) and the urea ($P < 0.001$).

Comparison of the results of Table 2 with those of Lofgreen, Loosli, and Maynard (1947) is of interest in view of the fact that these workers used rations comparable to our own in many respects, and especially in that the various nitrogen sources being compared comprised 40 per cent. of the total nitrogen of the rations. Their figures for urea, urea plus methionine, linseed meal, and whole dried egg were 71 ± 1.2 , 74 ± 1.8 , 76 ± 1.7 , and 80 ± 2.1 respectively. It is apparent that the first three of these are very similar to those of Table 2 but our figure for egg protein (86.7 ± 4.28) is appreciably higher and more variable.

No data are available with which our figure for the biological value of subterranean clover seed protein can be compared but there are a number for casein. Thus Harris and Mitchell (1941) obtained for casein a figure of 59 and Johnson *et al.* (1942, 1944) a figure of 60 for the growth of lambs. In both these cases the casein comprised 50 per cent. of the total nitrogen of the ration and the percentage total protein in the diets was higher than ours. This would tend to depress the biological values (Mitchell 1924) but even taking these factors into consideration, our figure of 82 ± 6.05 appears extremely high. It is possible that this is related, at least in part, to the nature of the casein preparation, since Reed, Moir, and Underwood (1949), using the same source of casein, obtained a biological value of 79 for the growth of rats. This is much higher than the figures of 62, 65, and 73 obtained by Olson and Palmer (1940), Hughes and Hauge (1945), and Beadles *et al.* (1933) respectively.

(b) Counts for Total Free Ruminal Bacteria

The outstanding characteristic of the figures for the total concentrations of ruminal bacteria, presented in Appendix II, is the marked individual variability between sheep on the same diet. The very high standard errors of the means given in Table 2, particularly for the linseed, casein, subterranean clover seed, and urea plus methionine rations, show this very clearly. This variability is difficult to explain in the present state of our knowledge, although a better understanding of the nature and cause of diurnal fluctuations, especially in relation to time and rate of food consumption, should throw considerable light on this problem. It should be pointed out, however, that marked individual fluctuation was shown by the mature wethers, as well as the lambs, although only those wethers were included in the results of Table 2 that had completely consumed their diets within 24 hours on all days previous to sampling and on sampling days had completely consumed their diets within six hours of feeding, i.e. before sampling.

Statistical examination of the mean results of Table 2 shows that the bacterial counts fall into two distinct groups. The first of these groups contains the counts from the urea plus methionine, casein, and egg rations. These do not differ significantly from each other but are all highly significantly greater ($P < 0.01$) than the counts of the second group. This group contains the urea, linseed meal, and subterranean clover seed rations, which again do not differ significantly from each other. The effect of the addition of methionine to the urea diet is very great—the mean count is raised from 17.7 ± 3.09 to 43.7 ± 9.12 million bacteria per cu. mm.

(c) Morphological Characteristics of the Bacteria

As accurate differential counts of the various organisms present were not made it is difficult to present the results of the morphological observations in a concise form. In Appendix III, however, an attempt is made to present the main features. In examining this it should be appreciated that morphological characteristics serve only as a guide to the predominating forms of microorganisms present.

On the urea, linseed, egg, and subterranean clover seed diets, most of the sheep showed a similar morphological picture for each diet but the sheep on the casein and urea plus methionine diets were less consistent. It appears that in these two groups the sheep were still showing the residual effects of previous dietary treatments. There is evidence that 24 days, which was the length of the treatments with our diets with the lambs, is not always sufficient to allow a proper expression of morphological changes induced by dietary means. Thus the results for wether IX (shown in Appendix III) indicate that the effect of green grazing, as evidenced by a high concentration of yeast-like forms (see Quin 1943; Van der Westhuizen, Oxford, and Quin 1950) can persist through two treatments. No other mature sheep or lamb had yeast-like forms present after 24 days on either the linseed or casein diets. In another experiment, yeast-like forms have been found in the rumen of one sheep after four months on a wheat gluten diet, whereas these organisms had entirely disappeared from its 19 companions under the same dietary conditions within 3-8 weeks.

A number of the mature sheep were continued on the various diets for a total of 40 days. During this extended period, on certain of the diets, a number of interesting changes in the balance of the morphological types of bacteria took place. These are indicated in Appendix III. It is important to note, however, that in no cases did the extra period of time on any of the diets result in any significant changes in the total concentrations of ruminal bacteria, compared with the 24-day period used for most of the sheep.

(d) Protozoal Counts

The individual protozoal counts are given in Appendix II both for the lambs and the mature sheep, but the mean figures for each treatment are not given in Table 2 because the variability shown, both between sheep on the same diet and between samples taken from the same sheep on different days, is so large that averages would, in most cases, be meaningless. No explanation of this tremendous variability can be given, although it can hardly be due to the counting technique or to the method of sampling, since protozoa are free movers in the rumen liquor. However, there appears to be no relationship between the diets fed and protozoal numbers.

IV. DISCUSSION

It is clear from the results given in the preceding section that different sources of nitrogen can vary significantly in their value for the promotion of nitrogen retention in growing lambs. To this extent our results are merely a confirmation and extension of the findings of Lofgreen, Loosli, and Maynard (1947) and in regard to the effect of methionine supplements in increasing the value of urea, also those of Loosli and Harris (1945). They are, however, in contrast to those of Johnson *et al.* (1942, 1944) and do not support the claim of these workers that with sheep "the biological value of the nitrogen of rations containing 10-12 per cent. crude protein ($N \times 6.25$) generally varies only within a few per cent. of 60." Nor do they support the implication of this claim that

the proportion of dietary nitrogen converted to bacterial protein is always high and relatively constant for all nitrogen sources. In fact the significant variation in the capacity of certain of our nitrogen sources to promote bacterial growth shows that there can be marked differences in this regard. If the concentration of ruminal bacteria is an indication of the extent of conversion of dietary nitrogen in the rumen to bacterial protein then it is obvious that this conversion was, with the linseed and subterranean clover seed diets, only about one-half that of the casein and egg diets. The difference was even greater with the urea plus methionine diet compared with the urea diet, in spite of the fact the only difference between the two rations was that the former contained a supplement of pure DL-methionine at a level of 0.2 per cent.

Although these results are quite definite, they are by no means easy to explain. However, it is of some interest to compare certain of the biological values obtained by us for lambs with those given in the literature for rats, although it must be recognized that in our work the test nitrogen made up only 40 per cent. of the total nitrogen. It can be assumed, for the purposes of preliminary argument, that these values would be much the same, if it were not for the intervention of the ruminal bacteria. The average biological value of the protein of flax seed (equivalent to linseed meal) is given by Block and Mitchell (1946) as 78 for growing rats. This is very close to our figure of 79.7 for growing lambs. It would appear that in this case the net effect of ruminal microflora on the nitrogen retention of the host has been negligible. This is not unexpected in view of the relatively low bacterial numbers on this diet. For the proteins of whole egg, Block and Mitchell (1946) give a biological value of 96 for the rat. This is 10 per cent. higher than our figure for this protein for lambs and 16 per cent. higher than that of Lofgreen, Loosli, and Maynard (1947). On this ration the numbers of bacteria in the rumen were high and their net effect appears to have been detrimental to the nitrogen balance of the host. This could have been due to such factors as preferential deamination of certain of the essential amino acids or loss by absorption of ammonia, owing to deamination of the egg protein by the bacteria more rapidly than it can be built up again into bacterial protein, and to the lower value of the bacterial protein.

The position with respect to the urea and urea plus methionine diets is slightly less complex because in these the supplementary nitrogen source is not directly available to the host. In our experiments the effect of the addition of methionine was to raise the mean concentration of ruminal bacteria from 17.7 ± 3.09 to 43.7 ± 9.12 million per cu. mm. and to increase the biological value of the nitrogen from 68.6 ± 1.52 to 75.2 ± 1.48 . It seems certain therefore that the improved nitrogen retention that occurs when methionine supplements urea as a source of nitrogen to the sheep can be largely explained by the methionine acting as a growth factor for the ruminal bacteria, resulting in greatly increased numbers of organisms and therefore greatly increased synthesis, from the urea, of bacterial protein. The possibility of some direct absorption of the methionine by the host, either from the rumen itself or from

lower parts of the digestive tract, cannot be excluded, however. This absorbed methionine would supplement the bacterial protein because this protein is somewhat deficient in methionine, as Reed, Moir, and Underwood (1949) have shown. It is suggested that any such supplementary effect must be small, compared with the effect of the methionine on bacterial growth and bacterial protein synthesis.

These results led us to examine the rest of the diets to see if the low bacterial numbers obtained with some of them could be explained by a low methionine intake. The approximate amounts of methionine contained in the six diets, calculated from the figures of Block and Bolling (1945) and Johanson and Lugg (1946), are included in Table 2. A comparison of these amounts with the mean concentrations of ruminal bacteria on the various diets suggests that lack of methionine has acted as a limiting factor on bacterial growth in the subterranean clover and possibly the linseed diets, as well as the urea diet. Examination of the individual data given in Appendix II, however, casts considerable doubt on this explanation because certain of the sheep in the linseed and subterranean clover groups showed concentrations of ruminal bacteria similar to those shown by most of the sheep in the groups with a high methionine intake. It is obvious that the relationship of methionine intake to bacterial growth in the rumen warrants further study. This problem, together with the possible significance of other essential amino acids, is under investigation in this laboratory at present.

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VI. REFERENCES

- BEADLES, J. R., QUISENBERRY, J. H., NAKAMURA, F. I., and MITCHELL, H. H. (1933).—*J. Agric. Res.* **47**: 947.
- BLOCK, R. J., and BOLLING, D. (1945).—"The Amino Acid Composition of Proteins and Foods." 1st Ed. (Ryerson Press: Toronto.)
- BLOCK, R. J., and MITCHELL, H. H. (1946).—*Nutr. Abstr. Rev.* **16**: 249.
- GALL, L. S., STARK, C. N., and LOOSLI, J. K. (1947).—*J. Dairy Sci.* **30**: 891.
- HARRIS, L. E., and MITCHELL, H. H. (1941).—*J. Nutrit.* **22**: 183.
- HUGHES, C. W., and HAUGE, S. M. (1945).—*J. Nutrit.* **30**: 245.
- JOHANSON, R., and LUGG, J. W. H. (1946).—*Aust. J. Exp. Biol. Med. Sci.* **24**: 321.
- JOHNSON, B. C., HAMILTON, T. S., MITCHELL, H. H., and ROBINSON, W. B. (1942).—*J. Anim. Sci.* **1**: 236.
- JOHNSON, B. C., HAMILTON, T. S., ROBINSON, W. B., and GAREY, J. C. (1944).—*J. Anim. Sci.* **3**: 287.
- LOFGREEN, G. P., LOOSLI, J. K., and MAYNARD L. A. (1947).—*J. Anim. Sci.* **6**: 343.
- LOOSLI, J. K., and HARRIS, L. E. (1945).—*J. Anim. Sci.* **4**: 435.

- MITCHELL, H. H. (1924).—*J. Biol. Chem.* **58**: 905.
- MOIR, R. J. (1951).—*Aust. J. Agric. Res.* **2** (3): 322.
- MOIR, R. J., and WILLIAMS, V. J. (1950).—*Aust. J. Sci. Res. B* **3**: 381.
- OLSON, F. C., and PALMER, L. S. (1940).—*J. Agric. Res.* **60**: 331.
- QUIN, J. I. (1943).—*Onderstepoort J. Vet. Sci.* **18**: 91.
- REED, F. M., MOIR, R. J., and UNDERWOOD, E. J. (1949).—*Aust. J. Sci. Res. B* **2**: 304.
- SOTOLA, J. (1930).—*J. Agric. Res.* **40**: 79.
- TURK, K. L., MORRISON, F. B., and MAYNARD, L. A. (1934).—*J. Agric. Res.* **48**: 555.
- TURK, K. L., MORRISON, F. B., and MAYNARD, L. A. (1935).—*J. Agric. Res.* **51**: 401.
- VAN DER WESTHUIZEN, G. C. A., OXFORD, A. E. T., and QUIN, J. I. (1950).—*Onderstepoort J. Vet. Sci.* **24**: 119.

APPENDIX I NITROGEN BALANCE RESULTS PER 10-DAY PERIOD

Sheep	Initial Weight (lb.)	Final Weight (lb.)	Average Weight (lb.)	Dry Matter Intake (g.)	Nitrogen Intake (g.)	Faeces Nitrogen (g.)	Metabolic Nitrogen (g.)	Nitrogen Urine (g.)	Endogenous Nitrogen (g.)	Apparent Digestible Nitrogen (%)	True Digestible Nitrogen (%)	Nitrogen Balance (g.)	Biological Value (%)	Digestible Dry Matter (%)
Diet A (Linseed)														
714	70	71	70.5	5392	83.0	34.2	21.6	23.9	10.7	58.5	84.7	+31.1	81.9	74.9
321	66	68	67	5841	89.9	26.0	23.4	29.9	10.2	71.1	97.1	+34.0	77.4	74.3
724	83	84	83.5	5841	89.9	32.6	23.4	26.8	12.7	63.7	89.7	+30.5	82.5	72.8
719	72	74	73	5151	77.8	33.9	20.6	23.1	11.5	56.5	83.2	+20.8	82.0	74.2
304	72	74	73	6500	107.7	38.3	26.0	32.3	11.1	64.4	88.6	+37.1	78.0	69.9
316	76	78	77	6500	107.7	35.9	26.0	37.8	11.7	66.7	90.8	+34.0	75.6	70.4
301	79	81	80	6500	107.7	33.8	26.0	32.9	12.2	68.6	92.7	+41.0	79.2	75.5
Diet B (Subterranean Clover Seed)														
304	58	61	59.5	5533	85.5	34.0	22.1	21.3	9.0	60.2	86.1	+30.2	83.3	65.9
725	69	70	69.5	5533	85.5	38.2	22.3	20.2	10.6	55.4	81.5	+27.2	86.2	69.6
723	59	64	61.5	5221	78.8	33.3	20.9	18.6	9.4	57.8	84.3	+25.9	86.0	69.5
721	76	79	78	5995	92.6	34.4	24.0	24.0	11.9	62.9	88.8	+34.3	85.2	70.0
301	80	81	80.5	6550	122.7	35.3	26.0	42.6	12.2	71.2	92.4	+44.8	79.0	70.3
312	65	68	66.5	6550	122.7	43.5	26.0	32.15	10.1	64.5	85.7	+47.1	79.0	68.5
316	72.5	76	74	6285	122.7	44.2	25.1	33.1	11.3	64.0	84.5	+45.5	79.0	60.8
714	69	72	70.5	5344	84.7	30.5	21.4	21.2	10.7	64.0	89.3	+33.1	86.2	73.5
Diet C (Powdered Whole Egg)														
719	67	71	69	4669	75.4	21.1	18.7	18.0	10.5	72.0	90.6	+36.3	89.7	70.7
724	73	77	75	5562	86.6	24.4	22.7	22.6	11.4	71.8	98.1	+39.7	86.8	79.8
722	68	69	68.5	4563	72.2	19.8	18.2	14.9	10.3	72.6	97.9	+37.5	93.7	77.4
312	59	61	60	5683	76.3	24.9	22.7	19.6	9.1	67.4	97.2	+31.9	85.9	76.0
306	54	56	55	5416	83.7	36.6	21.7	18.1	8.4	56.3	82.2	+29.0	87.2	65.7
321	77	78	77.5	6312	115.9	28.9	25.2	32.2	11.7	75.1	96.8	+54.9	81.0	73.2
318	77	79	78	6460	117.8	29.7	25.8	31.9	11.9	74.8	96.7	+56.1	82.4	74.3

APPENDIX I (Continued)

Sheep	Initial Weight (lb.)	Final Weight (lb.)	Average Weight (lb.)	Dry Matter Intake (g.)	Nitrogen Intake (g.)	Nitrogen Faeces (g.)	Metabolic Nitrogen (g.)	Nitrogen Urine (g.)	Endogenous Nitrogen (g.)	Apparent Digestible Nitrogen (%)	True Digestible Nitrogen (%)	Nitrogen Balance (g.)	Biological Value (%)	Digestible Dry Matter (%)
Diet D (Casein)														
724	77	79	78	5935	89.8	31.8	23.7	20.4	11.9	64.6	89.4	+37.6	89.4	76.1
725	63	65	64	5386	83.3	26.8	21.5	18.5	9.7	67.8	93.6	+37.9	88.7	76.7
301	64	66	65	5395	83.5	32.7	21.6	21.2	9.9	60.8	86.6	+29.5	85.7	71.8
312	58	60	59	5395	83.5	30.2	21.6	20.8	9.1	63.9	89.7	+32.5	85.4	75.6
321	72	76	74	6500	109.9	36.3	26.0	31.6	11.3	67.0	90.6	+42.0	79.5	76.9
316	70	73	71.5	6410	105.0	35.2	25.6	31.5	10.9	66.5	90.9	+38.3	77.0	68.9
314	71	73	72	6500	109.9	37.7	26.0	34.8	10.9	65.7	89.4	+37.4	76.0	72.3
304	79	80	79.5	6484	103.9	29.8	25.9	38.2	12.2	72.2	96.2	+35.9	74.0	77.4
Diet E (Urea)														
724	69	70	69.5	5436	84.9	24.9	21.7	36.4	10.6	70.8	96.4	+23.6	69.6	78.5
318	61	63	62	5911	92.3	33.0	23.6	36.0	9.4	64.2	89.9	+23.3	67.9	72.4
301	66	67	66.5	5911	92.3	30.9	23.6	37.3	10.2	66.5	92.1	+24.1	68.1	75.1
306	55	57	56	5911	92.3	33.9	23.6	36.0	8.5	63.3	88.9	+22.4	66.5	73.9
304	63	65	64	5911	92.3	30.6	23.6	36.4	9.4	66.8	92.4	+25.3	68.4	72.8
312	72	72	72	6500	114.5	31.3	26.0	42.6	10.9	72.6	95.3	+40.5	70.9	74.8
Diet F (Urea plus Methionine)														
725	72	73	72.5	5907	93.2	31.6	23.6	30.1	11.0	66.1	91.5	+31.4	77.6	74.6
316	65	68	66.5	5453	86.0	27.6	21.8	30.3	10.2	68.0	93.3	+28.2	75.0	73.2
322	62	64	63	5042	78.3	29.0	23.4	26.6	9.6	63.0	92.8	+22.7	75.5	70.1
301	74	76	75	6500	117.6	40.0	26.0	37.5	11.4	66.0	88.1	+40.1	74.8	73.9
318	73	75	74	6500	117.6	33.8	26.0	40.9	11.2	71.6	93.7	+43.3	73.0	74.1
321	78	80	79	6500	104.5	25.7	26.0	27.0	12.0	75.5	93.7	+51.8	75.0	76.9

APPENDIX III

QUALITATIVE MORPHOLOGICAL DESCRIPTIONS OF RUMEN BACTERIA (INCLUDING YEAST-LIKE FORMS)

Diet A	Diet B	Diet C
Linseed 24 days	Subterranean Clover Seed 24 days	Egg 24 days
Proportion of cocci to ovals and rods varies with individuals. Large oval $1.6 \mu \times 0.8 \mu$ always present. Large curved rod $3.5 \mu \times 0.5 \mu$ always present. Exception 1X — contained yeast-like forms with otherwise normal flora. Predominantly Gram —ve.	Majority predominantly rod flora. Rods mostly short $1.4 \mu \times 0.3 \mu$. Exceptions 5X, massed cocci, few rods. 714, Oval, coccal flora, few rods. Predominantly Gram —ve. 40 days 5X, Fewer cocci and more rods. Trend towards same as majority. Staining unchanged. 7X, Unchanged, in types and staining	A balanced cocci, oval, rod flora. Exception 724 — predominantly rods; straight, crescentic, and S-shaped. Predominantly Gram —ve.
4X, Staining and floral balance unchanged with time		
Diet D	Diet E	Diet F
Casein 24 days	Urea 24 days	Urea plus Methionine 24 days
Rod flora — 725, 2X, 3X. Rod, oval, coccal — 316, 314, 312. Cocci, oval — 301, 321, 304—small numbers rods. Coccal flora — 724, 1X — small numbers rods. 1X, Large numbers of yeast-like forms superimposed over coccal flora. Majority have conspicuous numbers of cocci (3 exceptions — 725, 2X, 3X). Predominantly Gram —ve.	Majority dominated by curved rods. Basal flora cocci. 306, Cocci — oval, few rods. 5X, Massed cocci — large curved rod. 6X, Massed cocci — few large curved rods. Predominantly Gram —ve. 40 days 6X, Curved rod nearly entirely gone. Massed cocci taken over	Ovals always high proportion of flora. Cocci as high as ovals in 4 cases. Rods as high as ovals in 2 cases. Predominantly Gram —ve.
1X, Yeast-like forms persisted in high numbers.		
Rods even smaller proportion of flora. Cocci taken over. Similar staining		

		725	724	723	722	721	719	714
Conc. rumen bacteria (millions per cu. mm.)	Sample 1		34				20	18
	Sample 2		28				20	20
	Average		31.0				20.0	19.0
Protozoa (No. per cu. mm.)	Sample 1		165				1248	153
	Sample 2		441				1336	153
Conc. rumen bacteria (millions per cu. mm.)	Sample 1	29		45		19		17
	Sample 2	32		44		16		16
	Average	30.5		44.5		17.5		16.5
Protozoa (No. per cu. mm.)	Sample 1	234		948		171		303
	Sample 2	243		630		192		315
Conc. rumen bacteria (millions per cu. mm.)	Sample 1		44		41		33	
	Sample 2		50		46		39	
	Average		47.0		43.5		36.0	
Protozoa (No. per cu. mm.)	Sample 1		1167		909		762	
	Sample 2		1236		291		276	
Conc. rumen bacteria (millions per cu. mm.)	Sample 1	32	57					
	Sample 2	29	43					
	Average	30.5	50.0					
Protozoa (No. per cu. mm.)	Sample 1	2448	1175					
	Sample 2	933	1011					
Conc. rumen bacteria (millions per cu. mm.)	Sample 1		18					
	Sample 2		18					
	Average		18.0					
Protozoa (No. per cu. mm.)	Sample 1		1251					
	Sample 2		1221					
Conc. rumen bacteria (millions per cu. mm.)	Sample 1	61						
	Sample 2	50						
	Average	55.5						
Protozoa (No. per cu. mm.)	Sample 1	977						
	Sample 2	450						

PROORGANISMS

	312	306	304	301	314	4X	6X	1X	5X	3X	2X	7X
			41	35		21		20		21		
			40	34								
			40.5	34.5		21.0		20.0		21.0		
			247	6								
			434	12								
eed)												
	18		20	24			21		44			29
	18		20	21								
0	18.0		20.0	22.5			21.0		44.0			29.0
	246		411	360								
3	258		435	1425								
	45	46										
	44	45										
	44.5	45.5										
	0	6										
	3	12										
	51		40	31	32			41		55	60	
	48		45	30	35							
5	49.5		42.5	30.5	33.5			41.0		55.0	60.0	
	45		1605	1620	162							
9	105		3438	1497	1221							
	19	12	12	19			20		20			
	19	13	13	19								
	19.0	12.5	12.5	19.0			20.0		20.0			
	1605	1500	630	180								
	891	1671	765	1376								
ne)												
				35								
				36								
.0				35.5								
2				21								
1				480								

CELL WALL ORGANIZATION AND THE PROPERTIES OF THE XYLEM

I. CELL WALL ORGANIZATION AND THE VARIATION OF BREAKING LOAD IN TENSION OF THE XYLEM IN CONIFER STEMS

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Summary

The variation of breaking load in tension of tangential longitudinal sections of wood, taken from successive growth rings of each of six conifer stems, has been studied. An increase in this property was observed in successive growth rings from the stem centre of each specimen. This was paralleled by an increase in tracheid length, basic density, and cellulose content. The inclination to the longitudinal cell axis of the spiral micellar system of the cell wall decreased with increasing tracheid length. Because of this known relation between spiral angle and tracheid length, the latter was used as an index of the spiral organization of the cell wall. The breaking load in tension of single tracheids taken from successive growth rings of two stems was also measured and found to increase with increasing cell length.

Failure in tension occurred in the cell wall. In specimens of late wood the line of failure followed the direction of micellar orientation of the middle layer of the secondary wall. This was also observed for early wood specimens although the line of failure was often irregular. Within the cell wall separation between the outer and middle layers of the secondary wall, and the outer layer of the secondary wall and primary wall, usually occurred during failure. This has been attributed to the differences in micellar orientation and the relative extent of the micellar and intermicellar systems of the adjacent cell wall layers.

It has been concluded that the breaking load in tension of the wood sections was governed by the cell wall organization of the constituent cells and by the basic density of the specimens. The increase in the breaking load in tension which occurred upon drying was attributed primarily to changes in the intercellular layer (middle lamella), because the breaking load of isolated tracheids was less in the dry than in the water-saturated condition.

It was demonstrated that an increase in radial growth rate of the stem resulted in a decrease in the average tracheid length. This was paralleled by a decrease in the breaking load in tension of the wood. The possible applications of the investigations in forest practice are briefly discussed.

I. INTRODUCTION

In this series of papers it is proposed to discuss the cell wall organization of the main tissue elements of wood in relation to the variation of its properties. The investigations described are confined for the most part to the study of variations in properties within conifer stems.

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Following earlier controversy the cell wall organization of conifer tracheids has now been established (Wardrop and Preston 1947) as being similar to that proposed originally by Kerr and Bailey (1934). The mature cell wall consists of two structures: the primary cell wall and the secondary cell wall. In the primary wall the micelles are oriented almost transversely to the longitudinal axis of the cell, but are considerably dispersed about this direction (Preston 1947; Preston and Wardrop 1949a). The secondary wall consists of three layers. In the outer and inner layers the micelles are spirally arranged, making an angle of $45-90^\circ$ to the longitudinal axis of the cell. The central layer also possesses spiral organization but the spiral angle is smaller, the micelles making an angle of between 0° and 45° with the longitudinal axis of the cell (Bailey and Vestal 1937; Wardrop and Preston 1947). The central layer is higher in cellulose content, and in late wood is considerably thicker than the other secondary cell wall layers. The intercellular substance (middle lamella) is optically isotropic and consists mainly of lignin with smaller amounts of hemicelluloses (Kerr and Bailey 1934).

The nearly transverse orientation of the micelles in the primary wall of conifer tracheids is independent of the cell length (Preston 1947), and of its lateral dimensions (Preston and Wardrop 1949a). However, in the secondary wall the angle of the spiral micellar orientation becomes less (i.e. the spiral becomes steeper) with increasing tracheid length. This was demonstrated quantitatively by Preston (1934, 1948) for the middle layer of the secondary wall of conifer tracheids, and has since been shown to hold for the outer layer of the secondary wall (Preston and Wardrop 1949b). Thus it is probable that the spiral organization of the entire secondary wall is determined by the cell length. The dependence of spiral angle on cell length has also been shown to hold for the abnormal tracheids of compression wood (Wardrop and Dadswell 1950), for the fibres of *Eucalyptus gigantea* Hook. f. (author's unpublished data), as well as for cotton fibres (Meredit 1946), and for the fibres of *Sansaviera* (Meeuse 1938). It is reasonable to suppose that the known anisotropy of wood properties is a reflection of its structure. The dependence of anisotropy of properties upon fibre structure has been demonstrated for textile fibres. In wood, however, the matter is complicated in that it is extremely difficult to predict, from the behaviour of a single cell, that of a group of cells aggregated to form a tissue. In this connection it is obvious that the nature of the intercellular substance would be of importance, depending upon the extent to which it is involved in governing adhesion and movement between cells.

However, insofar as cell wall organization does influence the anisotropy of wood properties it will be clear from the above discussion that so long as comparison is made within a stem a relation may be expected to exist between either the micellar spiral angle and the anisotropy of properties, or, because the cell length determines the micellar spiral angle, between cell length and the anisotropy of properties. Thus in material of similar origin the cell length can be regarded as a factor to which the anisotropy of properties may be related. Furthermore since cell length in conifer stems is determined by growth

conditions, a study of variation of properties with variation in cell length offers a means of relating growth factors to properties. From this standpoint the variation of tracheid length in conifer stems is of importance.

In a cross section of a conifer stem the tracheid length increases rapidly in successive growth rings from the pith, attaining a more or less constant value after 10-50 growth rings, and, in any one growth ring, increases from the base of the tree till it reaches a maximum some distance up the stem (Sanio 1872; for numerous other references see Bisset and Dadswell 1949). At any arbitrary level in a stem the relation between tracheid length and growth ring number can be expressed in simple mathematical terms (Wardrop 1948). The possible relation between tracheid length and rate of the radial growth of the stem has been discussed by Priestley (1930) who pointed out that rapid radial growth is effected by rapid tangential longitudinal division of the cambial initials. If this increased rate of tangential division involves a postponement of transverse divisions in the cambium it was suggested by Priestley that a wider ring would be associated with longer tracheids. If, on the other hand, an increase in the number of tangential divisions is accompanied by an increase in the number of transverse divisions, then a wider ring might be expected to be associated with shorter tracheids. The latter alternative has been demonstrated for compression wood (Wardrop and Dadswell 1950). Evidence supporting this conclusion for normal wood formation is contained in the present paper (Fig. 5). The tracheid length in conifer stems can thus be related to both the age and the rate of division of the cambial initials.

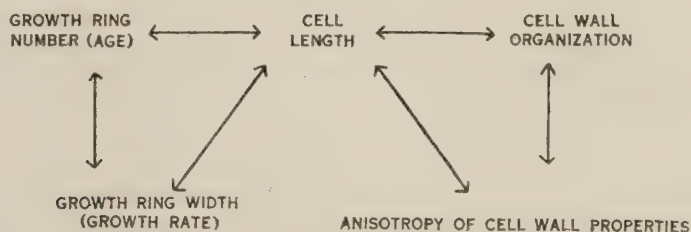


Fig. 1.—Some factors governing cell wall properties within a conifer stem.

The possible relations of the factors discussed above to the anisotropy of cell wall properties are illustrated in Figure 1. However, in specimens of wood, the magnitude of a property in a given direction will not depend solely upon the cell wall organization of the constituent cells, but may be modified or in some cases outweighed by other factors such as the basic density, gross anatomy of the specimen, or the nature of intercellular adhesion.

In the present series of investigations the influence of cell wall structure upon properties has been studied by following the variation in properties of small specimens of the xylem taken from comparable positions in successive growth rings from the pith, so that where possible in the one stem the influ-

ence of the gradual changes in cell dimensions and cell wall organization can be studied. This method possesses the advantage that genetically similar material is compared. In the first instance the investigations have been confined to gymnosperms because of their anatomical simplicity.

In the following investigation the variation of breaking load in tension has been studied, employing this general approach. The molecular factors involved in the rupture of fibres of cotton, flax, etc. have been extensively studied (see for example Hermans 1949) and it is reasonable to suppose that similar factors will also apply to conifer tracheids. Thus it is now generally agreed that failure in tension involves rupture of the primary valency chains rather than slipping between them, and that the degree of polymerization, chain length distribution, and the crystalline/non-crystalline ratio of the cellulose influence the properties (Mark 1943; Hermans 1949). Of major importance also is the angle of inclination of the micelles to the longitudinal axis of cells (Berkley and Woodyard 1938). Thus when a fibre with spiral micellar orientation is loaded in tension a compressive force is set up between adjacent regions of the micellar system. A shear force also acts between these regions, and the regions themselves are subjected to tensile forces acting parallel to the direction of orientation. Upon rupture of the cell in tension, separation of adjacent regions of the micellar system parallel to the direction of orientation occurs more readily when the inclination of the micelles to the longitudinal cell axis is large than when it is small. This is because the forces responsible for the lateral cohesion of the micellar system are considerably smaller than the primary valency forces responsible for the cohesion of the system parallel to its direction of molecular orientation.

The influence of these factors as well as the influence of basic density and various structural features upon the breaking load in tension are discussed in following sections of this paper.

II. MATERIALS

Cross sections from butt logs of the following plantation-grown conifers were used:

1. *Pinus radiata* D. Don—concentric, showing 18 growth rings—late wood of successive rings examined.
2. *Pinus radiata*—concentric, different from specimen 1, showing 18 growth rings—early wood of successive rings examined.
3. *Pseudotsuga taxifolia* (Poir.) Britt.—concentric, showing 18 growth rings—late wood of successive rings examined.
4. *Pinus radiata*—eccentric, showing 21 growth rings—early wood and late wood from the first 12 growth rings on the compression wood side examined and comparisons made with early wood and late wood from the same rings on the diametrically opposite side (rings 13-21 were rejected because of the presence of marked spiral grain).

5. *Pinus pinaster* Sol.—concentric, showing 31 growth rings—late wood of successive rings examined. The growth of the tree from which this section was taken had been greatly retarded but had responded remarkably to treatment with superphosphate. This was evident in the cross section, the first 15 growth rings being formed prior to the treatment.

6. *Pinus radiata*—concentric, showing 22 growth rings—late wood of successive rings examined. This tree had been suppressed and then released as a result of silvicultural treatment. The result was clearly marked in the cross section in which growth rings 10-16 from the pith were extremely narrow while growth rings 17-22 were very wide.

III. METHODS AND RESULTS

(a) Microscopic Examination of Tension Failures

Microscopic examination of the specimens in the region of failure was carried out after mounting the specimens in dilute glycerol. The main features of the tension failures from an early growth ring and from a later-formed growth ring of *Pinus radiata* are illustrated in Plates 1 and 2. Failures in both early wood and late wood specimens were examined.

In order to determine in what layers of the cell wall failure occurred, transverse sections ($20\ \mu$) were prepared from a standard tension specimen of *Pseudotsuga taxifolia* and examined between crossed nicols. The main features of failure can be seen in Plate 3, Figures 1, 2, and 3.

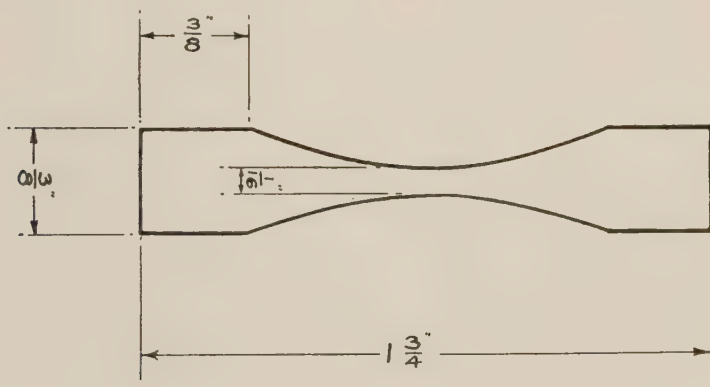


Fig. 2.—The lateral dimensions of the test sections of $80\ \mu$ in thickness used in the determination of breaking load in tension.

(b) Tension Tests on Longitudinal Tangential Sections

From each stem cross section a radial strip of wood was cut from the pith to the bark (in specimen 4 both on the compression wood side and the side diametrically opposite). Each strip was then shaped accurately on a

spindle moulder, so that the tangential faces appeared as indicated in Figure 2 with the dimensions shown. From the strips so prepared tangential longitudinal sections 80 μ in thickness were cut, using a sliding microtome, and kept wet until tested. By this means a number of serial sections could be obtained from early wood or late wood of successive growth rings as required for the various experiments.

TABLE 1
VARIATION OF TRACHEID LENGTH, BASIC DENSITY, MICELLAR SPIRAL ANGLE, AND
BREAKING LOAD IN TENSION OF 80- μ SECTIONS IN SUCCESSIVE GROWTH RINGS OF
PINUS RADIATA D. DON.

Specimen	Growth Ring No.	Tracheid Length (mm.)	Micellar Spiral Angle (degrees)	Basic Density (g./cc.)	Breaking Load in Tension (g.)	No. of Specimens Tested for Breaking Load
<i>Pinus radiata</i> late wood, specimen 1	2	2.53	25	0.500	558	6
	4	3.00	19	0.515	616	6
	6	3.65	16	0.465	850	6
	8	3.80	13	0.545	1137	8
	10	3.83	12	0.532	924	6
	12	4.07	11	0.550	1303	7
	14	4.22	14	0.575	1019	6
	16	4.38	10	0.515	1442	8
	18	4.31	11	0.625	1283	6
<i>Pinus radiata</i> early wood, specimen 2	1	2.81	41	0.275	394	12
	2	3.03	30	0.286	387	12
	3	3.58	24	0.298	297	12
	4	—	14	0.303	—	—
	6	—	14	0.326	389	12
	7	4.57	13	0.346	397	12
	8	3.68	15	0.363	484	12
	9	—	13	0.411	594	12
	10	4.14	15	0.403	642	12
	11	—	—	0.374	510	12
	12	4.39	14	0.359	595	12
	13	4.37	—	0.377	697	12
	14	—	14	0.402	781	12
	15	5.60	—	0.374	681	12
	16	—	14	0.423	795	12
	17	—	—	0.381	845	12
	18	5.36	11	0.458	868	12

These sections were used for investigating the breaking load in tension using the testing equipment developed in this laboratory (Kloot 1950). The number of sections tested for each growth ring depended on the width of the early wood or late wood bands being investigated. The results for the various specimens are given in Tables 1-3. The values so obtained are listed as the breaking load in g. per section. As only relative values for sections from different growth rings of the same stem cross section were required, no attempt was made to compute the corresponding stress.

(c) *Determination of Tracheid Length*

Half the sections from each portion of the growth ring under test were macerated (using hydrogen peroxide and glacial acetic acid) and the average length of 50 tracheids taken at random was determined. Results are given in Tables 1-4.

TABLE 2
VARIATION OF TRACHEID LENGTH, BASIC DENSITY, MICELLAR SPIRAL ANGLE, AND
BREAKING LOAD IN TENSION OF 80- μ SECTIONS IN SUCCESSIVE GROWTH RINGS OF
PSEUDOTSUGA TAXIFOLIA

Specimen	Growth Ring No.	Tracheid Length (mm.)	Micellar Spiral Angle (degrees)	Basic Density (g./cc.)	Breaking Load in Tension (g.)	No. of Specimens Tested for Breaking Load
<i>Pseudotsuga taxifolia</i> late wood, specimen 3	1	2.04	35	—	330	11
	2	2.27	28	0.448	745	16
	3	2.48	27	0.480	1199	11
	4	2.74	23	0.445	1158	16
	5	2.75	25	0.519	1197	16
	6	2.71	27	0.530	1449	16
	7	3.02	22	0.550	1492	16
	8	3.15	23	0.585	1511	12
	9	3.12	20	0.581	1365	12
	10	3.17	18	0.519	1353	12
	11	3.46	19	0.545	1430	16
	12	3.33	14	0.520	1343	16
	13	3.61	13	0.529	1511	12
	14	3.42	10	0.510	1543	16
	15	—	—	0.635	1654	12
	16	3.87	9	0.551	1388	16
	17	—	—	0.538	1388	12
	18	3.80	9	0.570	1358	8

(d) *Determination of Average Angle of Micellar Orientation in the Middle Layer of the Secondary Wall*

X-ray diffraction diagrams were obtained either from the actual test specimens or from carefully matched material, and the micellar spiral angle was determined from the spread of the 002 diffraction arc. $\text{NiK}\alpha$ radiation was used with a specimen-film distance of 3 cm. Results are given in Tables 1-3.

(e) *Basic Density*

The basic density of the wood from each growth ring was determined, using early wood or late wood as required by the experiment. For this purpose the wood was isolated from a radial strip adjacent tangentially to that used for the test specimens. The water-saturated volume of each piece from each ring was determined by the water displacement method and the dry weight obtained after drying for 2½ hours at 105°C. The data obtained are presented in Tables 1-3.

(f) *Radial Number of Cells per Growth Ring*

In specimens 5 and 6 transverse sections were cut from the centre of the stem to the bark and from these the number of tracheids in a radial row was

TABLE 3
VARIATION IN TRACHEID LENGTH, BASIC DENSITY, MICELLAR SPIRAL ANGLE, AND THE BREAKING LOAD IN TENSION OF 80- μ SECTIONS IN THE SUCCESSIVE GROWTH RINGS OF *PINUS RADIATA* D.DON (SPECIMEN 4)

Growth Ring No.	Tracheid Length of Late Wood (mm.)	Micellar Spiral Angle of Late Wood (degrees)	Basic Density (g./cc.)		Breaking Load (g.)		No. of Sections Tested for Breaking Load	
			Early Wood	Late Wood	Early Wood	Late Wood	Early Wood	Late Wood
Compression Wood Side of Stem								
1	1.59	43	0.379	0.483	266	261	69	10
2	1.77	—	0.402	0.489	—	400	—	6
3	2.08	45	0.391	0.491	—	414	—	20
4*	2.18	42	0.437	0.531	300	340	47	24
5*	2.49	42	0.475	0.522	—	445	—	13
6*	2.48	—	0.374	0.568	—	552	—	19
7*	2.61	38	0.476	0.568	436	585	34	17
8*	2.90	—	0.458	0.617	—	780	—	15
9*	3.10	33	0.477	0.694	513	872	14	18
10*	3.24	—	0.588	0.633	918	1110	14	13
11*	3.41	35	0.457	0.606	657	1139	25	13
12*	3.32	34	0.550	0.712	—	1073	—	6
Normal Wood Side of Stem								
1	1.44	46	—	0.436	—	278	12	12
2	1.75	—	0.398	0.462	224	356	—	11
3	2.02	41	0.371	0.391	—	386	—	13
4	2.30	37	0.406	0.452	—	336	50	8
5	2.67	40	0.359	0.505	227	669	—	11
6	3.02	—	0.352	0.545	—	961	—	8
7	3.07	33	—	0.515	—	881	60	8
8	3.32	—	—	0.545	647	1065	—	8
9	3.36	30	—	0.630	—	1387	—	6
10	3.63	—	—	0.660	—	1615	12	8
11	3.58	22	0.406	0.600	820	1238	50	25
12	3.57	—	0.431	0.550	878	1075	—	14

* Compression wood present.

determined for each growth ring. The results for specimen 6 (*Pinus radiata*) are given in Table 4 and Figure 6 and for specimen 5 (*P. pinaster*) are illustrated in Figure 5.

(g) Effect of Drying on Breaking Load in Tension

This was investigated with sections from the early wood of *Pinus radiata*. Longitudinal tangential sections, 80 μ thick, were cut as indicated above from four growth rings (Nos. 3, 6, 10, and 19). Half of the sections were selected at random from each growth ring and kept in water before testing. The remaining sections were oven-dried for two hours at 105°C. and tested immediately after removal from the oven. Results obtained from both the dry and wet specimens are shown in Table 5.

TABLE 4
INFLUENCE OF SILVICULTURAL TREATMENT BY THINNING UPON GROWTH OF
PINUS RADIATA D.DON (SPECIMEN 6)

Growth Ring No.	Tracheid Length (mm.)	Radial Number of Cells per Growth Ring	Breaking Load of Late Wood 80- μ Sections (g.)
1	1.64	421	—
2	1.86	356	242
3	2.43	363	—
4	2.61	316	276
5	2.97	240	331
6	2.95	222	—
7	3.09	129	502
8	3.34	110	—
9	3.25	108	—
10	3.46	106	624
11	3.76	91	—
12	3.46	80	—
13	3.26	68	687
14	4.01	56	1207
15	3.98	50	1098
16	3.92	66	1107
17	3.20	169	618
18	3.16	278	808
19	2.86	360	—
20	3.14	288	—
21	3.76	274	1131
22	3.46	145	1353

(h) Tension Tests on Isolated Tracheids

Sections cut from the early wood of successive growth rings of *Pinus radiata* were macerated either by means of the hydrogen peroxide and glacial acetic acid method or by the method of Cohen and Dadswell (1939). In preliminary experiments it was shown that if the former treatment were not too prolonged the fibre strength was not adversely affected. The variation in breaking load in tension of isolated tracheids from successive growth rings was investigated by the use of a modified Westphal balance. To one arm of the

balance a pan was suspended, holding a 100 ml. beaker. The weight of the pan and beaker was counterbalanced on the other arm. The ends of a single tracheid were attached by means of dental cement to pieces of fine silk thread,

TABLE 5
COMPARISON OF THE BREAKING LOAD IN TENSION OF SECTIONS ($80\ \mu$) OF EARLY
WOOD OF *PINUS RADIATA* D.DON, OVEN-DRY AND WATER-SATURATED

Growth Ring No.	Tracheid Length (mm.)	Breaking Load in Tension (g.)	
		Water-Saturated	Oven-Dry
3	2.45	300	596
6	3.16	473	1068
10	3.85	441	903
19	4.07	390	948

one of which was suspended from the balance arm holding the counterweight and the other attached to an adjustable metal rod set in a heavy base resting on the bench. By means of the adjustable rod the fibre and attached thread were drawn taut so that the balance arm was horizontal as indicated on the scale. Water was then allowed to flow into the beaker on the pan at a constant rate of 5.5 g. per minute until the tracheid failed. The water was then weighed.

TABLE 6
BREAKING LOAD IN TENSION OF EARLY WOOD TRACHEIDS ISOLATED FROM
DIFFERENT GROWTH RINGS OF *PINUS RADIATA* D.DON

Growth Ring No.	Tracheid Length (mm.)	Breaking Load per Tracheid (g.)	Cell Wall Area per Tracheid ($\text{mm.}^2 \times 10^{-4}$)	Breaking Load (kg./mm.^2)
1	2.33	2.40	3.07	7.8
3	3.17	3.73	3.80	9.8
6	4.11	5.34	4.42	12.1
10	4.67	6.27	5.40	11.6
13	4.83	7.09	6.42	11.0
17	5.13	5.96	4.57	13.0

By this means 20 tracheids from each growth ring were tested and results recorded when failure occurred in the centre of the tracheid away from the points of adhesion to the holding threads. An attempt was made to estimate the absolute value of the breaking load in tension by determining the average cross-sectional cell wall area per tracheid, from a cross section of the portions of the different growth rings from which the tracheids were isolated. The difficulty of obtaining accurate measurements in this manner was realized but it is of interest to consider the results obtained for different growth rings (see Table 6).

Using the above method the average breaking loads in tension of dry and wet tracheids from the same growth ring were determined. The results are given in Table 7.

TABLE 7
BREAKING LOAD IN TENSION OF CONIFER TRACHEIDS, WATER-SATURATED AND AIR-DRY

Specimen	Breaking Load per Tracheid (g.)		$\frac{BL_w}{BL_D}$
	Dry (BL_D)	Wet (BL_w)	
<i>Pinus radiata</i>			
early wood			
Ring 13	4.38	4.71	1.08
Ring 18	9.81	11.66	1.19

(i) Variation in Cellulose Content in Successive Growth Rings

The Cross and Bevan cellulose content was determined for early wood of successive growth rings in *Pinus radiata* (specimen 1) and for combined early wood and late wood of successive rings in *Pseudotsuga taxifolia* using the standard method of the Division of Forest Products. The values obtained were considered in relation to tracheid length and the angle of micellar spiral orientation of the middle layer of the secondary wall (θ). Results are shown in Table 8. The cellulose samples from the *P. radiata* growth rings were used in determining the moisture regain at 70°C. and 65 per cent. relative humidity.

TABLE 8
VARIATION OF CELLULOSE CONTENT OF WOOD FROM DIFFERENT RINGS OF CONIFER STEMS

Specimen	Growth Ring Number from Pith	Tracheid Length (mm.)	Micellar Spiral Angle (degrees)	Cross and Bevan Cellulose (%)	Moisture Regain (%)
<i>Pinus radiata</i> early wood	3	2.07	28	57.3	11.1
	4	2.69	21	58.7	11.2
	7	3.13	17	62.1	—
	10	3.51	12	62.5	10.8
	20	4.32	13	62.9	10.8
<i>Pseudotsuga taxifolia</i> early wood and late wood	2	1.6	31	58.0	—
	5	2.4	26	60.2	—
	10	2.9	15	62.5	—
	15	3.2	13	65.2	—
	20	3.5	12	65.3	—

IV. TREATMENT OF DATA

The variations in tracheid length, micellar spiral angle, breaking load, and basic density with successive rings from pith have been compared in Figure 3 for the late wood of *Pinus radiata* (specimen 1). The trends obtained are clearly shown. In Figure 4 the breaking load in tension has been plotted

against tracheid length, for the results obtained in the investigation of the successive growth rings of *P. radiata* (specimen 4, containing compression wood on one side).

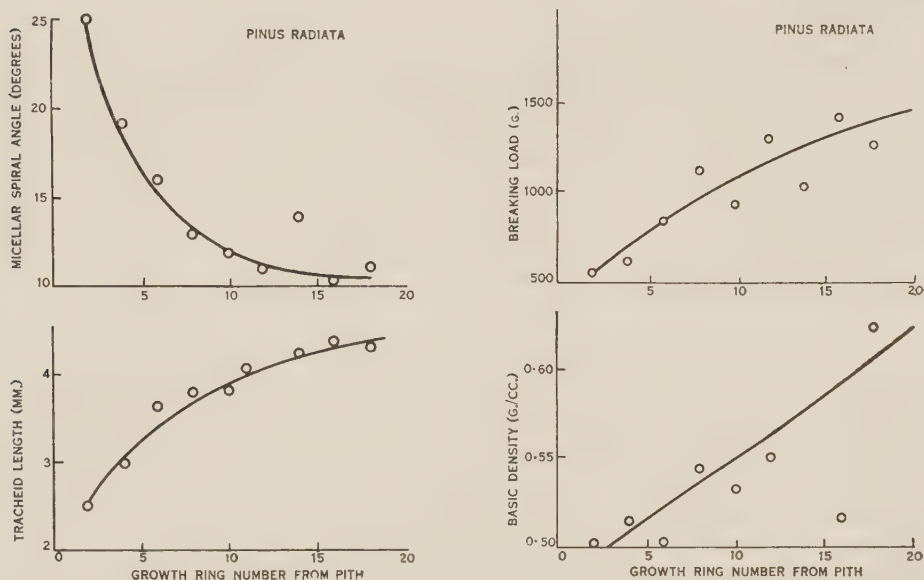


Fig. 3.—Variation of tracheid length, micellar spiral angle, basic density, and breaking load in tension in successive growth rings from the stem centre of *Pinus radiata* (specimen 1, late wood).

The results obtained with *Pinus pinaster* (specimen 5) and *P. radiata* (specimen 6), which were the two specimens showing the effect of suppression of radial growth and subsequent release, are shown in Figures 5 and 6. In Figure 5, tracheid length, micellar spiral angle, and number of cells per growth ring in the *P. pinaster* specimen have been plotted against growth ring number from the pith. Certain parallel and opposed relations may be noted. In Figure 6, tracheid length, number of cells per growth ring, and breaking load in tension for the *P. radiata* specimen have been plotted against growth ring number from the pith.

The relations obtained between breaking load in tension, BL_T (g. per section) and tracheid length, L (mm.) for the different specimens were as follows:

Specimen 1—*P. radiata* (late wood)

$$BL_T = 64.7 L^2 + 80 \quad (r = 0.914). \quad \dots \quad (1)$$

This gave a somewhat better fit to the data obtained than the straight line

$$BL_T = 474 L - 755, \quad \dots \quad (2)$$

although the improvement was not significant.

Specimen 2—*P. radiata* (early wood)

$$BL_T = 17.9 L^2 + 217 \quad (r = 0.757). \quad \dots \quad (3)$$

This relation did not give a significantly better fit than the straight line

$$BL_T = 151L - 90. \quad \dots \dots \dots (4)$$

Specimen 3—*Pseudotsuga taxifolia* (late wood)

$$BL_T = -4218 + 3360L - 496L^2 \quad (R = 0.867). \quad \dots (5)$$

Specimen 4—*Pinus radiata* (late wood from stem containing compression wood). It was found that a curve could be fitted to the data for both the compression wood side and normal side of the stem, viz.

$$BL_T = 922 - 789L + 254L^2 \quad (R = 0.961). \quad \dots \dots (6)$$

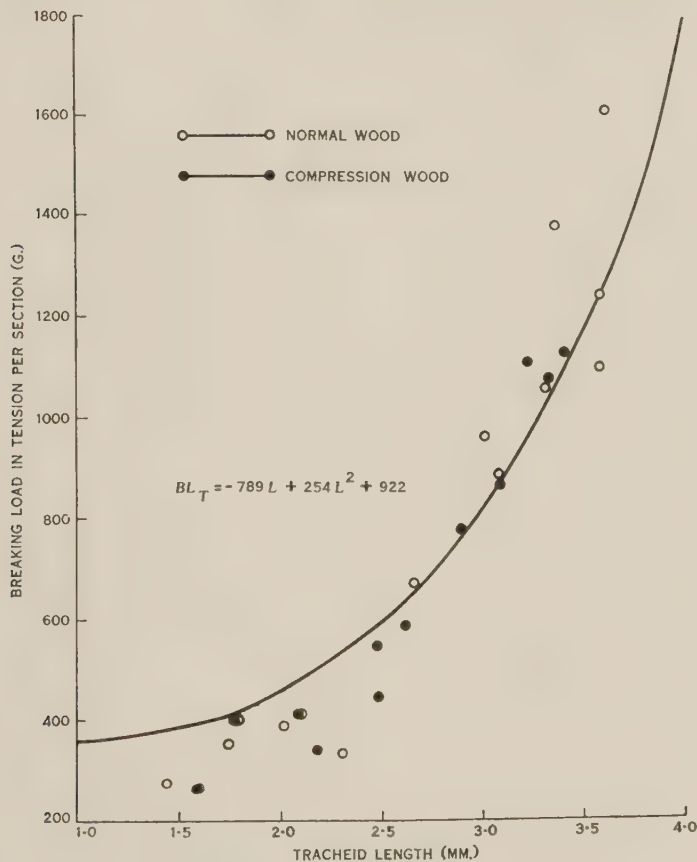


Fig. 4.—Relation between breaking load in tension (BL_T) and tracheid length (L) in *Pinus radiata* (specimen 4) containing both compression wood and normal wood.

From these relations it was apparent that the breaking load in tension and tracheid length were correlated (positively). However, from the specimens examined no conclusion could be drawn as to the form of the curves although, as will be pointed out below, a morphological interpretation of the trend of the values was possible in at least one case (specimen 3).

V. DISCUSSION

(a) The Anatomy of Tension Failures in Conifer Tracheids

With the exception of one doubtful case, in all the specimens examined during the present investigation, failure in tension of the wood sections involved rupture of the cell wall (Plate 1, Figs. 1-4; Plate 2, Figs. 1-6). That failure usually occurs within the cell wall has been reported elsewhere (Robinson 1921; Koehler 1933; Frey-Wyssling 1938; Garland 1939). This does not necessarily imply that the intercellular adhesion per unit area of contact surface between cells is greater than the strength of the tracheids, because the line of failure involved in the separation of cells is very much greater than that involved in the rupture of the cell walls.

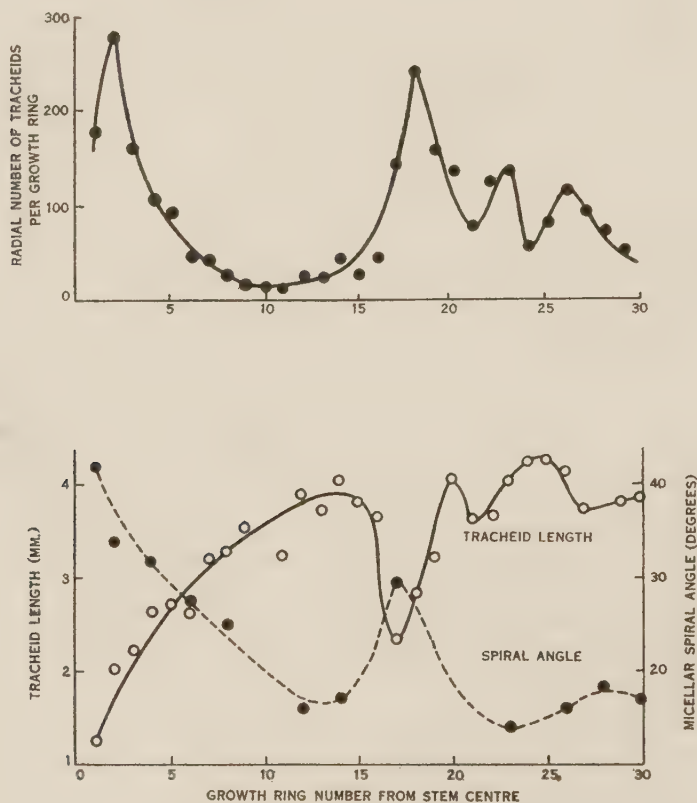


Fig. 5.—Variation of tracheid length, growth ring width, and micellar spiral angle in successive growth rings of *Pinus pinaster* (specimen 5).

Failure of the cell wall showed two features: (i) the rupture of the spiral micellar system in the different cell wall layers, and (ii) the separation of the different cell wall layers. In late wood the line of failure of the cell wall followed the direction of micellar orientation in the middle layer of the second-

dary wall. This is illustrated for a short tracheid in which the micellar spiral angle is relatively large in Plate 1, Figures 1 and 2, and in a long tracheid with a small micellar spiral angle in Plate 1, Figures 3 and 4. The fact that failure follows the direction of orientation in the central layer would indicate a relatively greater rigidity of this layer, which could arise from its greater thickness and higher cellulose content than the other cell wall layers. Parts of the primary wall can be seen adhering to the fractured tracheids in Plate 1, Figures

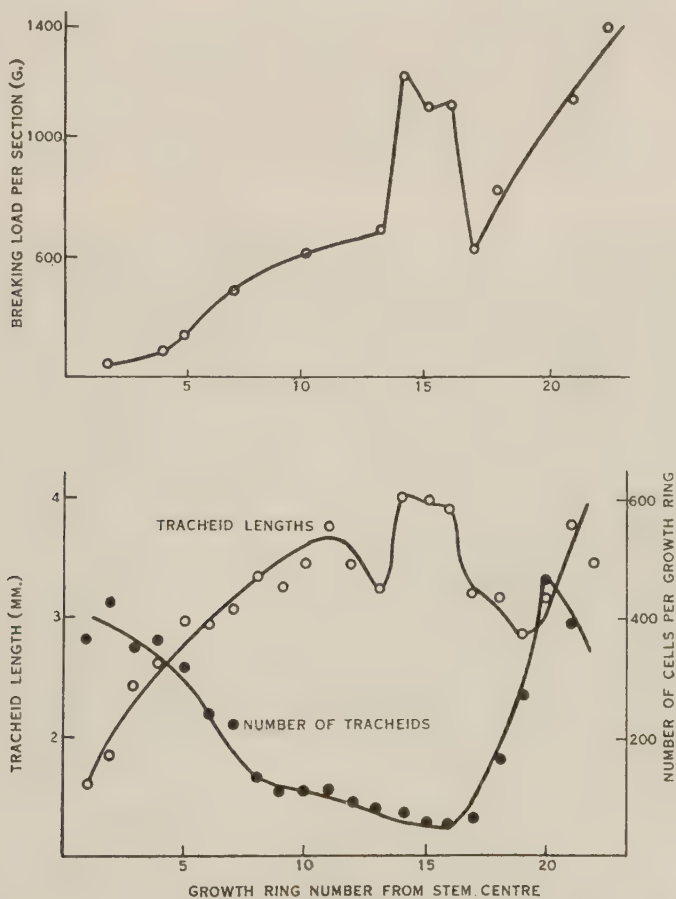


Fig. 6.—Variation of tracheid length, growth ring width, and breaking load in tension of the xylem from successive growth rings of *Pinus radiata* (specimen 6).

2 and 4. In early wood specimens the line of failure in the cell wall, as with the late wood, often followed the direction of micellar orientation in the middle layer (Plate 2, Figs. 1 and 2), but this was not always so and irregular failures were observed (Plate 2, Fig. 3). The irregular failure of early wood cells probably arises from the presence of a much thinner middle layer of the secondary wall, and the correspondingly greater influence of the outer and inner layers of the secondary wall and of the primary wall. Failure of the specimens was also frequently observed to occur in the rays (Plate 2, Fig. 4).

In tracheids of compression wood the line of failure also follows the direction of micellar orientation. In this case, however, unwinding of the micellar spiral system was easily observed (Plate 2, Fig. 5). This was due to the existence of radial discontinuities in the cell wall paralleling the direction of micellar orientation (Wardrop and Dadswell 1950). The same type of splintered failure perpendicular to the direction of spiral orientation was observed in all cases (Plate 1, Figs. 2 and 4; Plate 2, Fig. 6).

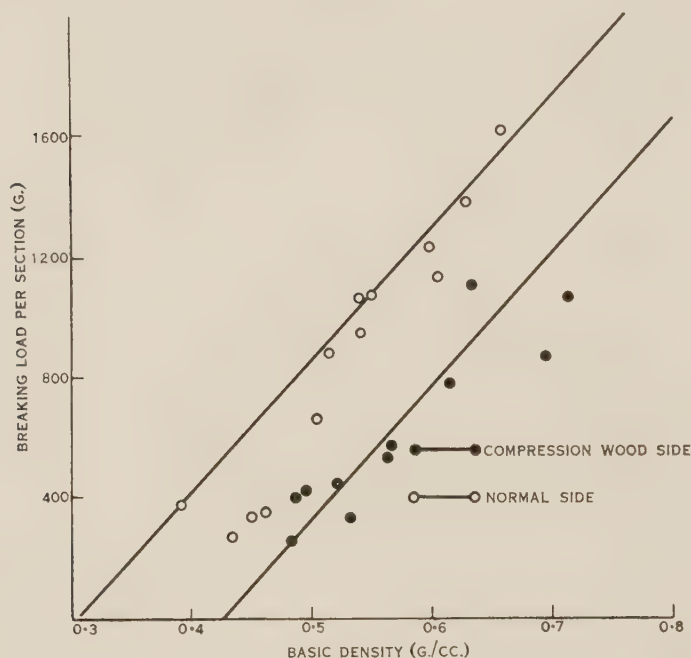


Fig. 7.—Relation between breaking load in tension and basic density of the xylem of *Pinus radiata* (specimen 4).

In the specimens examined in which separation of the cells was apparently involved it was often difficult to determine if true separation between cells was involved, or whether separation occurred between the different layers of the cell wall. In *Pinus radiata* failure between the primary and secondary wall was observed. This can be seen in Plate 1, Figure 5, in which fragments of the primary wall remained after separation of two tracheids. This was concluded to be the primary wall upon the basis of its extinction position (85° to the tracheid axis), birefringence, and dichroism after staining with congo red. In sections of *Pseudotsuga taxifolia* separation of tracheids appeared to have taken place (Plate 2, Figs. 7 and 8) although it was not possible to decide between which cell wall layers rupture occurred. This was examined further in transverse sections of a macro-tension specimen after failure had occurred. In Plate 3 separation of the primary wall and outer layer of the secondary wall (Fig. 3) and of the outer and central layers of the secondary wall (Figs. 2 and 3) can be seen. Consideration of the cell wall structure of conifer

tracheids suggests possible factors that may govern the adhesion between the different cell wall layers. Thus between the central and the outer and inner layers of the secondary wall there is a marked change in micellar orientation, as well as between the primary wall and outer layer of the secondary wall. In addition, the extent of the intermicellar system decreases enormously between the primary wall and the secondary cell wall, as well as between the outer and inner layers, and the central layer of the secondary wall. The great extent of the intermicellar system of the primary wall would tend to unify it with the intercellular layer so that failure might be expected to occur within the cell wall rather than between cells. Within the cell wall failure would, for similar reasons, be expected to occur more frequently between the outer and central layers of the cell wall than between the primary wall and outer layer of the secondary wall as well as because of the change in micellar orientation between these layers.



Fig. 8.—A diagrammatic representation of part of the cell wall of a conifer tracheid, in surface view with the cell axis parallel to the length of the page; illustrating the relative extents of the micellar and intermicellar systems (black). Successive layers of the cell wall are supposed to have been removed from right to left.

a, intercellular substance; *b*, primary wall; *c*, outer layer of the secondary wall; *d*, middle layer of the secondary wall; *e*, inner layer of the secondary wall.

An attempt to illustrate the relative extent of the micellar and intermicellar systems for the different regions of the cell wall is shown in Figure 8. The diagram was constructed assuming that the cellulose content of the outer layer of the secondary wall was approximately half that of the central layer (Lange 1949).

(b) Variation of Breaking Load in Tension of Wood within Conifer Stems

As pointed out in the Introduction of this paper, it was reasonable to expect that, upon the basis of their cell wall organization, the breaking load in tension of a short conifer tracheid in which the micellar spiral angle was large would be less than that of longer tracheids in which this angle was small. This was confirmed in the tests carried out upon single isolated tracheids from *Pinus radiata* (Table 6).

In determining the variation of the breaking load in tension of wood sections, measurements were attempted only in a direction parallel to the cell length, and because an accurate determination of the cross-sectional area of the specimens in the regions of failure was so laborious, attempts to calculate approximate values of the breaking stress were abandoned, although this was done for the tests involving single tracheids.

In view of these considerations, variations of the values of the breaking load in tension parallel to the tracheid length of the wood sections listed in Tables 1-4 and Figures 3, 4, and 6 could be attributed either to the changing orientation of the constituent tracheids or to changes in the density of the wood from which they were cut. For this reason the correlation coefficients between breaking load in tension and tracheid length, and between breaking load in tension and basic density, were determined. These values are listed in Table 9.

TABLE 9
LINEAR CORRELATION COEFFICIENTS BETWEEN BREAKING LOAD IN TENSION AND BASIC DENSITY, BREAKING LOAD IN TENSION AND TRACHEID LENGTH, AND TRACHEID LENGTH AND BASIC DENSITY

Specimen	Breaking Load and Tracheid Length	Breaking Load and Basic Density	Tracheid Length and Basic Density
<i>Pinus radiata</i> (specimen 1)	0.886**	0.631 (N.S.)	0.551 (N.S.)
<i>Pinus radiata</i> (specimen 2)	0.753**	0.902**	0.785**
<i>Pseudotsuga</i> <i>taxifolia</i> (specimen 3)	0.665**	0.715**	0.618*
<i>Pinus radiata</i> (specimen 4)			
Compression wood side	0.949**	0.859**	0.890**
Normal wood side	0.925**	0.920**	0.862**

N.S. = not significant.

** = significant at 1% level.

* = significant at 5% level.

In specimens 1 and 2, for which linear correlations between breaking load and tracheid length provide as good a fit for the data as curvilinear relations, it was found that the correlation coefficient between breaking load and basic

density was not significant for specimen 1 and in specimen 2 did not differ significantly from that for breaking load and tracheid length. In specimens 2, 3, and 4 there was a significant correlation between basic density and tracheid length (Table 9).*

Specimen 4, in which both normal wood and compression wood were present, provides further interesting information. Thus for both the normal wood side and the compression wood side of the stem, linear correlation coefficients between breaking load and basic density were found (Table 9) and the data could be represented by two linear relations (Fig. 7) as was found for specimens 2 and 3. However, comparing compression wood and normal wood it was found that, corresponding to any given value of the density (Fig. 7), there were two values of the breaking load significantly different from each other and the higher of which was that of the normal wood. Thus, although wood of higher basic density did possess a higher breaking load than wood of lower basic density for both the normal wood and compression wood sides of the stem, when comparison was made between these groups the density was no longer necessarily a criterion of breaking load.

In contrast to this, when the relation between breaking load in tension and tracheid length was examined it was found that a single relation (equation 6 above and Fig. 4) served to describe the data for both normal wood and compression wood. Thus both with the compression wood and normal wood groups, and between these groups, a small tracheid length served as a criterion of low breaking load and a larger tracheid length of a higher breaking load. These considerations confirm the previous general conclusion (Wardrop and Dadswell 1950) that comparison of the properties of compression wood in relation to normal wood should be made between compression wood and normal wood composed of tracheids of equal length (i.e. between cells of similar spiral cell wall organization) in order to eliminate the influence of structural differences between the constituent cells.

In the specimen of *Pseudotsuga taxifolia* examined (specimen 3) it will be noted (Table 2) that the breaking load in tension did not continue to increase after the eighth growth ring (tracheid length = 3.15 mm.) although the tracheid length did continue to increase. This was found to be due to failure occurring either between cells or between cell wall layers as discussed above (Plate 2, Figs. 7 and 8). Once the condition was reached that failure no longer occurred within the cell wall any further increase in the length of the constituent cells and corresponding decrease in their micellar spiral angle made no additional contribution to the strength of wood, although presumably the strength of the individual cells did increase.

Thus of the specimens examined, both the cell wall organization and the basic density of the specimens 2 and 3 appeared to have been of importance

* It may be noted further that in a recent investigation Kraemer (1950) found higher correlation coefficients between micellar spiral angle and the modulus of rupture and modulus of elasticity than between specific gravity and these properties in *Pinus resinosa*.

in influencing the value of the breaking load in tension observed. In specimens 1 and 4, however, the influence of cell wall organization appeared to predominate.

A further factor probably influencing the breaking load is tension in the cell wall composition. In Table 8 it can be seen that for both *Pinus radiata* and *Pseudotsuga taxifolia* the cellulose content of the xylem increased in successive rings from the pith and was apparently correlated with both tracheid length and micellar spiral angle. Although the increase in cellulose content was small it may have a significant effect upon the breaking load because relatively long cells with a higher cellulose content would have more cellulose chains potentially capable of taking an applied load in tension than shorter cells with a lower cellulose content. As the micellar spiral angle was smaller in the longer cells, not only were there more chains but they were more favourably oriented to take an applied load in tension. The possible influence of the cellulose content upon the intrinsic density of the cell wall substance would not be great because of the small variation in composition and the density of cellulose is but slightly higher than that of other cell wall constituents (Stamm and Hansen 1937).

It is possible that the correlation between cellulose content and spiral micellar organization as suggested by the data in Table 8 is of more general application. Thus within a single cell wall of a tracheid or fibre the cellulose content of the middle layer of the secondary wall is higher than that of the outer layer whereas the spiral angle is smaller. A similar correlation has been observed for different phloem fibres (Wardrop and Preston, unpublished data).

(c) *Change of Breaking Load in Tension with Moisture Content*

From Table 5 it can be seen that the breaking load in tension of the wood sections increased on oven-drying. This phenomenon is well known in wood (Forsyth 1944; Stamm 1936) and in phloem tissue (Haberlandt 1928). Stamm (1936) attributed the general increase in strength on drying to an increase in fibre strength in the following terms: "The strength of a swollen fibre in general increases upon drying. This is explained on the basis of the secondary valence forces between the micelles which in the swollen condition are partially satisfied by mobile water being brought together on drying thus satisfying each other."

If this explanation is correct it can be seen that the strength of individual tracheids of the wood should be greater in the dry than in the water-saturated condition. However, in a number of textile fibres the tensile strength has been observed to be greater in the wet than in the dry condition, and in the present study this has been demonstrated for conifer tracheids (Table 7). This increase in strength has been attributed to the water present in the intermicellar regions of the wet fibres permitting movement to occur between adjacent cellulose molecules so that a more uniform distribution of internal stress is achieved upon loading (Hermans 1949). In contrast to these results it has been reported by Klauditz, Marschall, and Ginzl (1947) that pulp fibres

possess a higher tensile strength in the dry condition, although it will be apparent that in this latter case considerable chemical breakdown had probably taken place.

If it is accepted that the tensile strength of single tracheids is greater in the wet condition, it is clear that the explanation by Stamm for wood quoted above cannot hold, and some other factor must be operative which results in a decrease in tensile strength of the wood with increasing moisture content predominating over the simultaneous increase in the tensile strength of the individual cells. It is suggested, therefore, that the two factors governing the strength of wood at any moisture content are the cell wall organization and the intercellular substance. In contrast to the behaviour of the tracheid the amorphous intercellular substances would be expected to weaken with increasing moisture content and consequent swelling, so that at least an increase in the plasticity of this layer could be expected. Thus in spite of any increase in strength of the tracheids, because of the influence of the intercellular substances, a decrease of the breaking load in tension could be expected with increasing moisture content.

This view receives some support from the observation that failure in tension of oven-dry specimens appears macroscopically regular and involves uniform rupture of the cell wall (Plate 3, Fig. 4), whereas tension failures in water-saturated specimens have a splintered appearance macroscopically, which results from failure taking place between cells in parts of the section, although fractures of many cell walls were still involved (Plate 3, Fig. 5). A similar observation has been made by Frey-Wyssling (1938) and by Garland (1939). Further support for the hypothesis presented above may be found in the observation of Kollman (1944) that a maximum tensile strength for wood exists at 8 per cent. moisture content, although apparently this maximum has not been observed in investigations of other static properties. However, if the breaking load in tension is determined by the interaction of the above factors, it is possible that if the intercellular layer weakens continuously with increasing moisture content, and the breaking load of the tracheids increases to a constant value as does cotton at 9 per cent. moisture content (Brown, Mann, and Peirce 1930), then a maximum might be expected to occur when the moisture content approached this value, which is close to that (8 per cent.) at which a maximum value of the breaking load in tension was observed by Kollman.

(d) Variation of Breaking Load in Tension with Radial Growth Rate

As pointed out in the Introduction, within any one stem a large radial growth rate (number of cells per growth ring) might be expected to involve the formation of short tracheids, and a small radial growth rate the formation of longer tracheids. This was found to be the case in the two specimens examined; in one, specimen 5, the growth rate was stimulated after the formation of 15 growth rings by the addition of superphosphate, in the other, specimen 6, after the formation of 16 growth rings by silvicultural treatment. In

both cases it is apparent from the results obtained (see Figs. 5 and 6) that the increase in radial growth rate arising from the treatments was accompanied by a decrease in tracheid length.

From an examination of Figure 6 a general parallelism of the trends observed for tracheid length and the breaking load in tension of wood from successive growth rings can be seen, the longer tracheids corresponding to higher values of breaking load as observed in other specimens examined. These results, although of only a preliminary nature, thus serve to give some indication of the way in which the breaking load in tension could be expected to change with varying radial growth rate and suggest an advantage that would be gained by maintaining an even radial growth rate of the stem.

It has also been observed (Wardrop 1948) in the examination of different trees of the same species from the same locality, that in a specimen in which the tracheid length of the first growth ring was large, the tracheid length continued to be greater in subsequent growth rings than in a specimen in which the tracheid length of the first growth ring was small. This observation suggests that if, by suitable nursery treatment or selection, seedlings could be obtained with initially long tracheids, then all the subsequently formed wood would consist of long tracheids. Insofar as a large value of tracheid length can be correlated with a large value of breaking load in tension (and preliminary experiments have shown similar correlations for other properties), it can be seen that the development of stems of high initial tracheid length may offer a means of effecting an improvement in the quality of the wood subsequently formed. Such an increase in tracheid length would also be advantageous in timber used for pulping.

It is fully realized that the latter conclusions are based on limited evidence, but they are presented here with a view to providing a starting point from which the implications of the present studies, in relation to the conditions governing development of conifer stems, may be further investigated.

VI. ACKNOWLEDGMENTS

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VII. REFERENCES

- BAILEY, I. W., and VESTAL, M. R. (1947).—*J. Arnold Arbor.* 18: 186.
BERKLEY, E. E., and WOODYARD, O. C. (1938).—*Industr. Engng. Chem. (Anal. Ed.)* 10: 451.
BISSET, I. J. W., and DADSWELL, H. E. (1949).—*Aust. For.* 13 (2): 86.

- BROWN, K. C., MANN, J. C., and PEIRCE, F. T. (1930).—*J. Text. Inst. Manchr.* **21**: 187.
- BUSGEN, M., and MUNCH, E. (1929).—"Structure and Life of Forest Trees." (Chapman and Hall: London.)
- COHEN, W. E., and DADSWELL, H. E. (1939).—*J. Coun. Sci. Industr. Res.* **12**: 115.
- FORSAITH, C. C. (1944).—In Wise, L. E., "Wood Chemistry." (Reinhold: New York.)
- FREY-WYSSLING, A. (1938).—Schweiz. Verb. Mater. Prof. Tech. Ber. Nr. **36**: 29.
- GARLAND, H. (1939).—*Ann. Mo. Bot. Gdn.* **36** (1): 1.
- HABERLANDT, G. (1928).—"Physiological Plant Anatomy." p. 164. (Macmillan: London.)
- HERMANS, P. H. (1949).—"The Physics and Chemistry of Cellulose Fibres." (Elsevier: New York.)
- KERR, T., and BAILEY, I. W. (1934).—*J. Arnold Arbor.* **15**: 327.
- KLAUDITZ, W., MARSHALL, A., and GINZEL, W. (1947).—*Holzforsch.* **1**: 98.
- KLOOT, N. H. (1950).—C.S.I.R.O., Melbourne. For. Prod. Newslett. No. 181: 3.
- KOEHLER, A. (1933).—Causes of brashness in wood. U.S. Dep. Agric. Tech. Bull. No. 342.
- KOLLMAN, F. (1944).—*Naturwissenschaften* **32** (14/26): 121.
- KRAEMER, J. H. (1950).—*J. For.* **48** (12): 842.
- LANGE, P. (1949).—First Internat. Congr. Biochem. Abstr. 433/11.
- LUDTKE, M. (1946).—*Melliand's textilber.* **27**: 283. (*Chem. Abstr.* **43**: 4858(d).)
- MARK, H. (1943).—In Ott, E., "Cellulose and Cellulose Derivatives." (Interscience: New York.)
- MEEUSE, A. D. J. (1938).—*Rec. Trav. Bot. Néerland* **35**: 288.
- MEREDITH, R. (1946).—*J. Text. Inst. Manchr.* **37** (9): T 205.
- PRESTON, R. D. (1934).—*Philos. Trans. B* **224**: 131.
- PRESTON, R. D. (1947).—*Proc. Roy. Soc. B* **134**: 202.
- PRESTON, R. D. (1948).—*Biochim. Biophys. Acta* **2**: 370.
- PRESTON, R. D., and WARDROP, A. B. (1949a).—*Biochim. Biophys. Acta* **3**: 549.
- PRESTON, R. D., and WARDROP, A. B. (1949b).—*Biochim. Biophys. Acta* **3**: 585.
- PRIESTLEY, J. H. (1930).—*New Phytol.* **19** (1): 56.
- ROBINSON, W. (1921).—*Philos. Trans. B* **210**: 49.
- SANIO, K. (1872).—*Jb. wiss. Bot.* **8**: 401.
- STAMM, A. J. (1936).—Colloid chemistry of cellulose materials. U.S. Dep. Agric. Misc. Publ. No. 240.
- STAMM, A. J., and HANSEN, L. A. (1937).—*J. Phys. Chem.* **41**: 1007.
- WARDROP, A. B. (1948).—Proc. Ninth Annu. Coop. Res. Conf.: 62. (C.S.I.R.O.: Melbourne.)
- WARDROP, A. B., and DADSWELL, H. E. (1950).—*Aust. J. Sci. Res. B* **3** (1): 1.
- WARDROP, A. B., and PRESTON, R. D. (1947).—*Nature* **160**: 911.

EXPLANATION OF PLATES 1-3

PLATE 1

- Fig. 1.—*Pinus radiata*. A late wood tracheid, from the third growth ring from the stem centre, broken in tension. Note the flat spiral fracture ($\theta = 50^\circ$). $\times 430$.
- Fig. 2.—The same tracheid as in Figure 1 in different focus, showing the serrated failure perpendicular to the direction of spiral fracture. $\times 430$.
- Fig. 3.—*P. radiata*. A late wood tracheid from the eighteenth growth ring from the centre of the same stem as that in Figure 1, showing failure at a slip plane. $\times 430$.
- Fig. 4.—*P. radiata*. A late wood tracheid similar to that in Figure 3, showing the type of failure accompanying very steep spiral orientation (cf. Figs. 1 and 2). $\times 430$.
- Fig. 5.—*P. radiata*. A late wood tracheid showing portion of the primary wall of an adjacent cell which had separated during failure in tension. $\times 430$.

PLATE 2

- Fig. 1.—*Pinus radiata*. Early wood tracheids after failure in tension in which the failure paralleled the micellar orientation of the middle layer of the secondary wall. x430.
- Fig. 2.—Similar to Figure 1 except that separation between cells has also occurred. x430.
- Fig. 3.—Similar to Figures 1 and 2 but failure in the cell wall is here irregular. x430.
- Fig. 4.—Similar to Figures 1-3, and illustrating failure at a wood ray. x430.
- Fig. 5.—*Pinus radiata*. A compression wood tracheid after failure in tension, illustrating the unwinding of the spiral structure due to the presence of radial discontinuities in the cell wall. x430.
- Fig. 6.—Similar to Figure 5, illustrating the serrated failure perpendicular to the direction of micellar orientation. x980.
- Fig. 7.—*Pseudotsuga taxifolia*. A group of tracheids separated during failure in tension apparently without involving rupture of the cell wall. x100.
- Fig. 8.—The tip of the terminal cell of Figure 7. x430.

PLATE 3

- Fig. 1.—*Pseudotsuga taxifolia*. Transverse section of the xylem extending over a region of failure in tension. Photographed between crossed nicols. x430.
- Fig. 2.—Similar to Figure 1, illustrating failure between the cell wall layers. Crossed nicols. x430.
- Fig. 3.—Similar to Figures 1 and 2 illustrating failure along a radial file of tracheids. Crossed nicols. x430.
- Fig. 4.—*Pinus radiata*. A tension failure in a section of early wood ($80\ \mu$) after drying. x100.
- Fig. 5.—*P. radiata*. A tension failure in a water-saturated section of early wood ($80\ \mu$). x100.

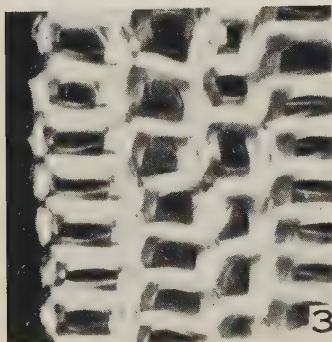
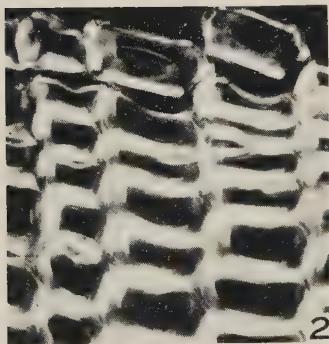
CELL WALL ORGANIZATION AND XYLEM PROPERTIES. I



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TERTIARY ARAUCARIACEAE FROM SOUTH-EASTERN AUSTRALIA, WITH NOTES ON LIVING SPECIES

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Summary

The foliage shoots, male cones, and female cone-scales of a new Tertiary species of *Araucaria*, section *Eutacta*, from the brown coal at Yallourn, Victoria, have been described; leaves and male and female cones possessing the general features that characterize species of *Agathis* have been identified. Leaves of a second species of *Agathis* have been distinguished in a bed associated with the brown coal at Bacchus Marsh, near Melbourne, Vic.

Pollen grains from fossil male cones of *Araucaria* and *Agathis* have been recognized and the exine structure of a recent species of *Araucaria* has been considered. Fossil pollen grains of araucarian type have also been recorded from several deposits in south-eastern Australia and Tasmania.

The morphological and cuticular features of the leaves, male cones, and female cone-scales of recent species of *Araucaria*, section *Eutacta*, and *Agathis* have been investigated.

The present and past southern distribution of both genera have been discussed.

I. INTRODUCTION

There is not a great deal of reliable information available regarding the exact identity of Australian Tertiary conifers. The comprehensive review of southern Mesozoic and Tertiary conifers published by Florin in 1940 includes all Australian records existing at that date, and indicates the author's views on the accuracy of their identification. More recently Krausel (1949) revised the determinations of fossil coniferous woods, and drew up a key for their separation.

About half of the recorded Australian species apparently belong to the family Podocarpaceae, most of the remainder being members of the Araucariaceae or Cupressaceae. However, many of these determinations are open to question, particularly those based on leaf morphology. Much of the confusion surrounding species identified from wood fragments has been eliminated by Krausel, who at the same time has considerably reduced the number of distinct Australian species. Few cones or seeds have been recorded, and the accuracy of the identification of some of these has been questioned by Florin.

As a result of visits to the Yallourn open cut during the last few years, a collection of coniferous remains has been built up. This consists of reproductive organs and leaves sufficiently well preserved to show microscopic features.

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It is hoped that this material will supply more details than have hitherto been available, and thus permit more precise identifications. Supporting evidence obtained from the palynological examination of Tertiary deposits is also being collected, and it is proposed to publish the results of these combined studies as opportunity offers. The present communication, which is concerned with the Araucariaceae, provides conclusive proof of the presence of the genera *Agathis* and *Araucaria* in Victoria during the Tertiary period.



Fig. 1.—Map of south-eastern Australia, showing the locations in which Tertiary members of the Araucariaceae occurred (prepared by the Geological Survey of Victoria).

II. SCOPE OF THE INVESTIGATION

Most of the specimens to be considered here were collected from the State Electricity Commission's open cut at Yallourn, Vic. A small number from a sandy leaf-bed above the coal at the Lucifer Colliery near Bacchus Marsh, Vic., and from other Australian deposits (see Fig. 1), will also be discussed.

The fossils include:

- (a) Leafy shoots, detached male cones with pollen grains, and isolated female cone-scales typical of *Araucaria* (section *Eutacta*).
- (b) Leaves of two species of *Agathis*; one detached male cone with pollen grains and two female cones which are also believed to have belonged to *Agathis*.
- (c) Pollen grains of araucarian type from Tertiary sediments in south-eastern Australia.

A detailed study of the leaves, male cones, pollen grains, and female cone-scales of living species of *Araucaria* and *Agathis* has been undertaken with a view to establishing the generic identity and specific affinities of the plant remains listed above. This has shown that a number of small variations exist between the species of both genera. Unfortunately it has not been possible, from the limited amount of authentic material available, to determine the constancy of the individual variations. However, while realizing that a more comprehensive examination may necessitate future modifications in some directions, it has been decided to publish the observations made in this connection. These are shown in Tables 1-5.

III. LOCATION AND AGE OF THE DEPOSITS

The location of the sediments in which pollen grains of the Araucariaceae have been observed is shown in Figure 1. Most of these deposits have been mentioned previously (Cookson 1946, 1947, 1950). New localities are:

Bingera, New South Wales. Pink mudstone, Australian Museum. Probably Oligocene (Singleton 1941).

Berwick, Vic. Ligneous clay underlying the older basalt at Wilson's Quarry. Probably Oligocene.

Hamilton, Vic. Ligneous clay on the north side of Grange Creek. Military map grid reference Hamilton sheet 496,346. This deposit underlies the newer basalt, and Mr. E. D. Gill, Palaeontologist to the National Museum of Victoria, has personally expressed the view that its age is Upper Pliocene.

Ouse, Tasmania. Lignitic seam in the Ouse River, about 400 yards upstream from the Bridge hotel, Ouse. ? Oligocene-Miocene.

The age of the brown coal deposits in south-eastern Australia has been the subject of considerable discussion. The latest statement is that of Thomas and Baragwanath (1949), who accept the view that the brown coals are mainly of Oligocene age.

IV. METHODS

For the removal of cuticles from leaves and sporophylls of both fossil and recent types, treatments either with nitric acid and potassium chlorate followed by ammonia, or with a 12 per cent. solution of sodium hypochlorite were used.

Pollen grains were obtained after maceration of portions of the male cones by sodium hypochlorite or by Erdtman's (1948) chlorination-acetolysis-alkali

method. For the recovery of pollen grains from ligneous clays and shales, the latter method, after a pretreatment with hydrofluoric acid, proved to be most satisfactory.

V. ARAUCARIA, SECTION EUTACTA

Portions of leafy shoots of a species of *Araucaria* (Section Eutacta) were relatively abundant in a bed exposed during 1950 at the third level of the Yallourn open cut, the coordinates of which were 7160S. and 1050E. and the reduced level 92. With them were disconnected male cones and detached female cone-scales which also resemble those of members of the section Eutacta. Although no organic connection between the three types of fossil has been observed, their relative abundance and close proximity in this particular zone make it seem likely that they represent different organs of one species. As such they are viewed here, where all three are included in the new Tertiary species *A. lignitici* now to be described.

(a) Leaves

Araucaria lignitici n.sp.

(i) External Characters (Plate 1, Figs. 1-4)

The leaves (presumed to be adult) are arranged on the slender axes in a rather close spiral. They are from 3 to 8 mm. long and from 1 to 1.5 mm. broad, usually narrow-triangular with an acute apex, falcate, and prominently carinate. In the small fragment shown in Plate 1, Figure 3, the leaves are shorter and relatively broader.

(ii) Cuticular Structure (Plate 1, Figs. 5, 7)

Stomata.—The leaves are amphistomatic, but the arrangement of the stomata differs on the two surfaces. The stomata are relatively large, the average polar diameter of the guard cells being $55\ \mu$ and the range $47\text{--}60\ \mu$. The guard cells are surmounted by from four to six rather inconspicuous subsidiary cells, one or two of which are frequently polar, and a similar number of large, clearly defined, encircling cells. Usually the stomata are separated from one another by ordinary epidermal cells, but sometimes the encircling cells of neighbouring stomata abut on one another. The majority of the stomata are obliquely oriented.

Lower epidermis.—The epidermal cells are square, quadrangular, polygonal, or irregular, with their major axis in a longitudinal direction. Their lateral walls are straight or curved, $2.5\text{--}3\ \mu$ thick, and pitted, often inconspicuously so.

The stomata are arranged in more or less well-defined longitudinal rows situated in two roughly triangular areas, which are separated from one another by a wide central zone completely devoid of stomata. Two lateral zones without stomata also occur.

The stomatal rows, which at the base are about 12 in number, become gradually reduced to one on either side towards the middle of the leaf. Soon

afterwards the two remaining rows are discontinued, to recommence at varying distances from the apex, where they are represented by from 6 to 20 stomata. This arrangement (type B2, Fig. 2), shown in Plate 1, Figure 5, has been observed in a considerable number of leaves, and appears to be the one typical of the species. Slight variations have been noted in the extent of the gap between the proximal and distal rows, which in some examples may be quite small. An exception to this arrangement was seen in a leaf removed from the specimen with shorter, broader leaves shown in Plate 1, Figure 3. In it the stomata appeared to be restricted to two proximal groups as in type C, Figure 2. More material of this type will be necessary before the significance of this exception can be determined.

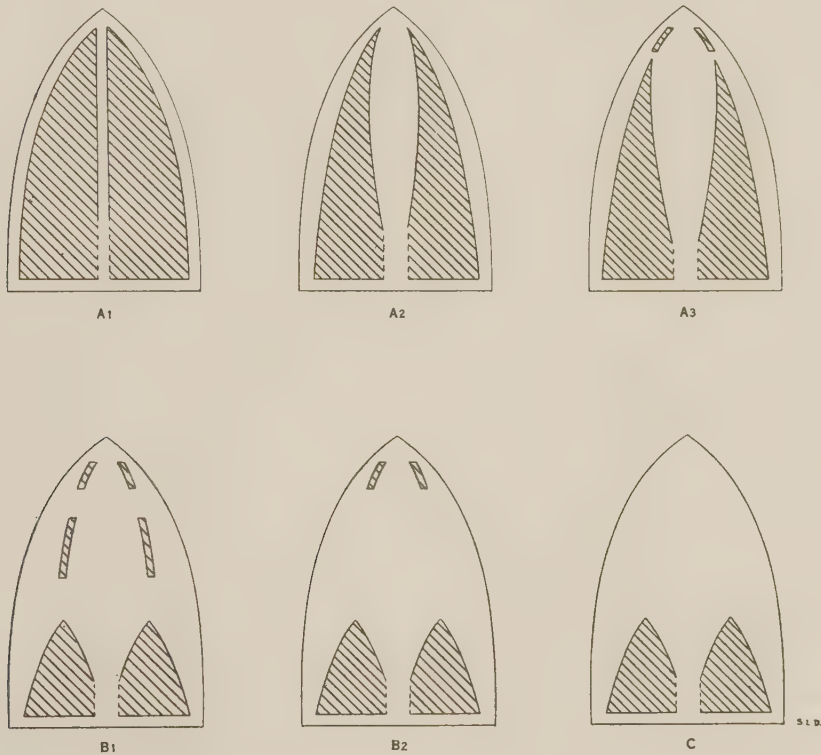


Fig. 2.—Diagrammatic representation of the types of stomatal arrangement found in recent species of *Araucaria*, section *Eutacta*.

Upper epidermis.—The stomata are more closely placed than on the lower epidermis. They occur in longitudinal rows. Towards the base of the leaf there are usually from six to eight rows on either side of a wide, central, stomata-free zone. The number of stomatal rows becomes gradually reduced distally, but the few that remain extend without appreciable interruption from base to apex. Two lateral zones without stomata occur.

The epidermal cells are similar in all respects to those of the lower epidermis.

(iii) *Internal Anatomy* (Plate 1, Fig. 6)

The internal tissues are rather compressed, and only the gross features can be distinguished. The mesophyll is differentiated into spongy and palisade tissues, and branched sclereids and resin canals occur.

The cells of the epidermal layer are small, square to quadrangular in cross section, and have thick ($5\ \mu$), cutinized outer walls. Beneath it on both surfaces is a hypodermal layer composed of thick-walled fibres interrupted only at the stomata. The guard cells (Plate 1, Fig. 6) are deeply sunken, their outer walls extremely thick ($7.5\ \mu$), and their cavities considerably reduced. They are almost completely covered by the subsidiary cells, which themselves lie vertically beneath the encircling cells, so that a funnel-like outer chamber is formed.

Recent Species

Of the conifers still living in Australasia and the south Pacific islands, only *Athrotaxis selaginoides* Don, and certain species of *Dacrydium* and *Araucaria* show any morphological resemblance to *Araucaria lignitici*. Comparisons with a view to the establishment of the affinity of this fossil have, therefore, been restricted to these particular types.

The similarity between *A. lignitici* and *Athrotaxis*, apparent as regards external morphology, does not extend to the cuticle. In *Athrotaxis* the stomata are irregularly arranged and are restricted to the upper surface of the leaf, whereas in *Araucaria lignitici* they are arranged in rows and occur on both surfaces of the lamina. Moreover, the stomatal apparatus is different in the two forms, that of *Athrotaxis* being without the encircling cells that are a conspicuous feature in *Araucaria*. The leaves of such species as *Dacrydium araucarioides*, *D. balansae*, and *D. lycopodioides* can be distinguished from those of *A. lignitici* by their cuticular structure. In these forms (group B, Florin 1931, p. 248) the stomata are always longitudinally oriented and the walls of the subsidiary and encircling cells associated with them are more or less conspicuously ribbed.

On the other hand, the evidence of general morphology, cuticular structure, and internal anatomy indicates a close affinity with the genus *Araucaria*.

Two sections are recognized within this genus, namely *Colymbea* and *Eutacta*. The species included in the section *Colymbea* need not be considered further, because of their relatively large leaves and the distinctive cuticular features, such as the predominantly longitudinal orientation of the stomata, that characterize them. Moreover, comparative work has clearly demonstrated that the shoot of *A. lignitici* is very similar to those of certain species of the section *Eutacta* — *A. balansae*, *A. beccarii*, *A. cunninghamii*, and *A. excelsa*. Confirmation of such a taxonomical position has been obtained from a comparative study of 11 species of this section. The observations made, together with information provided by Bertrand (1874), Thomson (1905), Seward and Ford (1906), Florin (1931), and Dallimore and Jackson (1948) are contained in the summary and discussion which follow, and in Table 1.

(i) *External Characters*.—The leaves of living species of *Eutacta* are small (*A. muelleri* excepted), sessile, either lanceolate, triangular, or ovate, and have acute or obtuse apices. They are usually four-angled except when considerably flattened, spirally and imbricately inserted, and upwardly and inwardly curved.

(ii) *Cuticular Structure*.—Stomata occur on both sides of the leaf. On the upper surface they are arranged in rows that form two triangular areas extending from the base, where they may join, to a position just behind the apex. These areas are separated by a median zone of variable width devoid of stomata.

The stomata are less closely placed on the lower surface, and the size of the triangular areas is more variable. Three main types of stomatal arrangement can be distinguished (Fig. 2).

(A) The stomata occupy two triangular areas, which extend to, or nearly to, the apex.

(A1) The areas are separated only by a very narrow zone of epidermal cells.

(A2) The width of the stomatal areas decreases very considerably towards the apex, and they are separated by a relatively wide zone of epidermal cells.

(A3) As for A2, but there is a short gap between the stomata at the apex and those just behind it.

(B) The stomata are mainly concentrated in two triangular areas in the proximal region of the leaf, but isolated groups occur at or near the apex.

(B1) Isolated groups of stomata occur between the apical and basal groups.

(B2) The apical and basal groups are completely separate.

(C) The stomata are confined to two basal, triangular areas.

These types of stomatal arrangement, although described as being separate, actually form a continuous series. It has been found that, while the stomatal arrangement of a species may not always conform to one particular type, variations from it in either direction are only slight.

The stomata are usually obliquely oriented and have polar diameters of from 44 to 72 μ . Each consists of two deeply sunken guard cells surrounded and surmounted by from four to six subsidiary cells and an equal number of encircling cells. The walls that separate the subsidiary cells from the encircling cells are particularly strongly cutinized. The outer respiratory cavity is either oval or rectangular in surface view.

The epidermal cells are more or less elongated in a longitudinal direction. Their lateral walls are straight, curved, or sinuous, and are usually pitted.

(iii) *Internal Anatomy*.—The leaves are from flat to elliptical or more or less tetragonal in cross section. The outer walls of the epidermal cells are considerably thickened and a cryptocrystalline zone, consisting of minute grains of calcium oxalate, is present beneath the cuticle. The guard cells of the stomata

TABLE 1
MORPHOLOGICAL AND CUTICULAR CHARACTERS* OF THE LEAVES OF RECENT AND
FOSSIL SPECIES OF *ARAUCARIA*, SECTION *EUTACTA*

Species	Leaf		Cuticle									
			Cell Walls			Stomata						
	Size (mm.)		Sinuous		Pitted		Group (See Section V(a)(iv))	Diameter (μ)	Range			
										Av.	Polar	Range
	Length	Width	Upper Surface	Lower Surface	Upper Surface	Lower Surface						
<i>A. balansae</i> Brongn. et Gris.	4-6	2-4	+	-	+	+	B1, B2	55	50-61			
<i>A. beccarii</i> Warb.	c. 10	c. 3	+	+	+	+	A2, A3	50	45-55			
<i>A. bernieri</i> Buchh.	c. 3.5	c. 2	+	-	+	+	B1, B2	60	54-68			
<i>A. biramulata</i> Buchh.	8-9	4-6	+	-	+	+	A2, A3	58	54-67			
<i>A. columnaris</i> (Forst.) Hook.	5-6	c. 4	+	×	+	+	C	51	48-57			
<i>A. cunninghami</i> Ait.	c. 6	c. 4	+	+	+	+	A3, B1, B2	48	45-55			
<i>A. excelsa</i> (Lamb.) R. Br.	c. 6		+	-	+	+	B2, C	47	44-49			
<i>A. humboldtensis</i> Buchh.	6-7	4-5	-	+	+	+	C	64	54-72			
<i>A. intermedia</i> Vieill.†	c. 13	c. 6	+	+	+	+	B2, C	62	52-70			
<i>A. montana</i> Brongn. et Gris.	c. 7	c. 5	+	×	-	-	B2	54	45-59			
<i>A. muelleri</i> Brongn. et Gris.	25-30	12-15	-	+	+	+	A1	63	56-68			
<i>A. rulei</i> F.v.M.‡	12-26	6-8	-	+	+	+	A1	54	45-68			
<i>A. lignitici</i>	3-8	1-1.5	-	+	+	+	B2	55	47-60			
<i>A. balcombensis</i> Selling	c. 32	c. 16	-	-	?	?	B1, B2, ?C	?	?			
<i>A. derwentensis</i> Selling	c. 7	c. 5	?	?	?	?	?	?	?			
<i>A. fletcheri</i> Selling	c. 4.5	c. 3	-	-	-	-	?	?	?			
<i>A. ruei</i> Seward and Conway	c. 12	c. 7	×	-	-	-	?	?	?			

* In Tables 1-5, + and — are used to indicate presence or absence of the character listed at the top of any particular column, and × to denote an intermediate condition. Most of the figures relating to the size of leaves and cones have been taken from Seward and Ford (1906), Bailey and White (1916), Dallimore and Jackson (1948), and Buchholz (1949). Measurements from actual specimens have been used only when figures from such sources have not been available. The cell walls referred to in the tables are the lateral walls of the epidermal cells. They are regarded as being sinuous only when the undulations are conspicuous, of a narrow amplitude, and closely placed. The walls are classed as thick when they are of the order of 5 μ, thin if about 2 μ.

†, ‡ see page 423.

are deeply sunk beneath the surface of the epidermis. A fibrous hypodermal layer is present. The palisade and spongy mesophyll form two distinct layers and the latter is characterized by the presence of numerous thick-walled, branched sclereids. Resin canals are either scattered irregularly or may occur below each vein; in those leaves that are tetragonal in section there is frequently a canal at each angle.

Comparison of Araucaria lignitici with Recent and Fossil Species

Recent species.—In external form, the leaves of *A. lignitici* resemble most closely those of *A. balansae*, *A. beccarii*, *A. cunninghamii*, and *A. excelsa*, those of the remaining species differing from them either in size or shape. The type of stomatal arrangement usually found on the lower surface of the leaves of *A. lignitici* (Fig. 2, B2) appears to be typical of *A. balansae*,* *A. bernieri*, and *A. montana*, and has also been observed in *A. cunninghamii*, *A. excelsa*, *A. intermedia*, and *A. rulei*.* A somewhat similar type (Fig. 2, A3) has been recorded in *A. beccarii* and *A. biramulata*, but in these species the main stomatal areas extend much nearer to the apex than they do in *A. lignitici*. The distribution of the stomata on the leaves of *A. columnaris* (type C), *A. humboldtensis* (type C), and *A. muelleri* (type A1) appears to be quite distinct from that of *A. lignitici*.

In size, the stomata of *A. lignitici* approximate closely to those of recent species (Table 1), the average polar diameter of the guard cells being well within the range of averages (44–72 μ) obtained for the stomata of living representatives. The stomata of *A. excelsa*, which are consistently smaller, and those of *A. humboldtensis*, *A. intermedia*, and *A. muelleri*, which are usually larger, are the farthest removed in this respect.

Another feature that may prove useful for comparative purposes is the nature of the lateral walls of the epidermal cells. These are straight or curved on both surfaces of *A. lignitici*, *A. balansae*, *A. bernieri*, *A. biramulata*, *A. excelsa*, *A. muelleri*, and most specimens of *A. rulei*, but are sinuous on at least one surface of the material of *A. rulei* ex Kew and of the remaining species.

From the above discussion and the details shown in Table 1, it can be seen that the leaves of *A. lignitici* have more features in common with the leaves of *A. balansae* than with those of any other recent species. Whether this resemblance of foliar characters indicates a close relationship cannot be determined from the present evidence.

* According to Dallimore and Jackson (1948), the stomata of *A. balansae* and *A. rulei* are confined to the upper surface of the leaf. In all the material examined, stomata have always been present on both surfaces of both species.

† The cuticular characters of a single leaf of *A. intermedia* Vieill. No. 1276 ex Kew are included here although Dallimore and Jackson's mention of a "Var. *intermedia* Intermediate in foliage between *A. rulei* and *A. columnaris*" seems to imply their intentions to reduce this form to varietal rank.

‡ Two sets of information regarding the cuticular characters of *A. rulei* appear in this table. The upper one refers to material from all sources (Appendix I) except the Royal Botanic Gardens, Kew, the lower one refers to the specimen received from that institution.

Fossil species.—Four reliable species of the section *Eutacta** have previously been described from Tertiary deposits in the Southern Hemisphere. They are *Araucarites ruei*, described by Seward and Conway (1934) from shoots and cone-scales found at the Kerguelen Archipelago, and the three species, *Araucaria derwentensis* from Tasmania, *A. balcombensis* from Victoria, and *A. fletcheri* from New South Wales described by Selling (1950) from sterile shoots and isolated leaves.

As many comparative features as possible have been extracted from the original descriptions and included in Table 1. These show that each of the southern fossil species is distinct from *A. lignitici* in more than one feature.

Possibly *A. lignitici* resembles *A. fletcheri* more closely than it does any of the other three species, but its leaves are narrower, its epidermal cells have pitted instead of unpitted lateral walls, and its stomata are not "closely packed" as they are said to be in *A. fletcheri*.

(b) Male Cones

Araucaria lignitici

(i) External Characters (Plate 2, Figs. 8-11)

Portions of detached male cones resembling those of recent species of *Araucaria*, section *Eutacta*, were relatively numerous in the particular locality at Yallourn that yielded shoots of *A. lignitici*.

As already indicated, none of the male cones of *A. lignitici* is complete, and most are considerably compressed. They appear to have been cylindrical, with a length of about 2 cm. and a width of approximately 0.5 cm.

The sporophylls are numerous, small, rather larger towards the base of the cone, and spirally arranged. Each consists of a stalk and a thin, flat, rhomboidal, vertically directed free portion, or lamina, with an acute or acuminate apex. There are several pollen-sacs per sporophyll, but the exact number has not been discernible.

The base of the cone is surrounded by a number of sterile bracts (Plate 2, Fig. 9), about 3-4 mm. long and 0.5 mm. broad, that are slightly carinate and somewhat falcate.

(ii) Cuticular Structure of the Lamina of the Sporophyll (Plate 2, Fig. 13)

Lower epidermis.—The epidermal cells are square, quadrangular, polygonal, or irregular, and elongated in a longitudinal direction. Their lateral walls are curved or slightly sinuous, 2.5-4 μ thick, and strongly pitted.

The number and arrangement of the stomata vary according to the position of the sporophyll on the cone, being few and widely spaced on, or absent from, the smaller terminal sporophylls, and more numerous, with a tendency to form short, longitudinal rows, in the central areas of the larger basal sporophylls.

* *Araucaria imbricatiformis* R. M. Johnston is omitted because of the disagreement existing regarding its sectional position. Johnston (1888) and Florin (1940) relate it to the section *Colymbea*, but Selling (1950) writes: "It seems to come closest to the recent *A. muelleri*, a species of *Eutacta* restricted to the higher elevations in New Caledonia."

The stomata are identical with those of the leaves of *A. lignitici*. Each is associated with the usual number of subsidiary and encircling cells. Their orientation is variable.

Upper epidermis.—The epidermal cells are rather more regular and elongated than those of the lower surface, and their lateral walls are thinner and less strongly pitted. Stomata are absent.

Margin.—The margin of the lamina is minutely crenulate. Most of the marginal cells develop bluntly pointed, frequently recurved, cylindrical denticulations, the walls of which are about $2\ \mu$ thick.

(iii) *Cuticular Structure of the Bract* (Plate 2, Fig. 12)

Lower epidermis.—The epidermal cells are usually six-sided and longitudinally elongated; they have thin (about $2.5\ \mu$), straight, strongly pitted lateral walls. Stomata appear to have been almost completely absent, only a single short row having been observed at the proximal end.

Upper epidermis.—The epidermal cells are similar to those of the lower surface, but their walls are less strongly pitted. Stomata of the normal type occur in two more or less unbroken rows, which extend from the base to the apex of the bract. These rows are separated from one another by a wide, central, stomata-free zone, and from the margin by two narrower zones in which stomata do not occur.

Margin.—Some of the cells at the junction of the upper and lower epidermis are prolonged into short, unicellular denticulations similar to those of the sporophylls.

Comments.—The distribution of the stomata on the lower surface of the bract as compared with that of the foliage leaves and sporophylls is of interest. The stomata on the lower surface of the foliage leaf are mainly in the proximal half of the leaf, with a few short rows at the apex. The same surface of the bract is almost devoid of stomata, only one or two occurring towards the base. In the sporophyll, however, the distribution of the stomata is reversed, a moderate number usually being scattered over the central area of the lower surface of the lamina, while there are none on the upper surface.

The development of minute denticulations from some of the marginal cells indicates the transitional character of the bracts. Marginal denticulations are numerous in the laminae of the male sporophylls, less numerous in the bracts, and undeveloped in the foliage leaves of *A. lignitici*.

(iv) *Pollen Grains* (Plate 2, Figs. 14, 15)

Pollen grains of araucarian type have been recovered from the male cones of *A. lignitici*, both after chlorination-acetolysis and treatment with sodium hypochlorite. The morphological features of the grains are equally clearly shown in both cases, but the measurements given have been taken from acetolysed grains.

The pollen grains of *A. lignitici* are non-aperturate and, when fully expanded, circular in outline. The average diameter is $59\ \mu$, and the range $52\text{--}68\ \mu$.

The exine is approximately $2\ \mu$ thick, and is clearly differentiated into nexine and sexine layers (Erdtman 1948) of almost equal thickness. The sexine is composed of numerous, densely arranged, oval granules or pilae about $1\ \mu$ long. Each terminates in a minute spine.

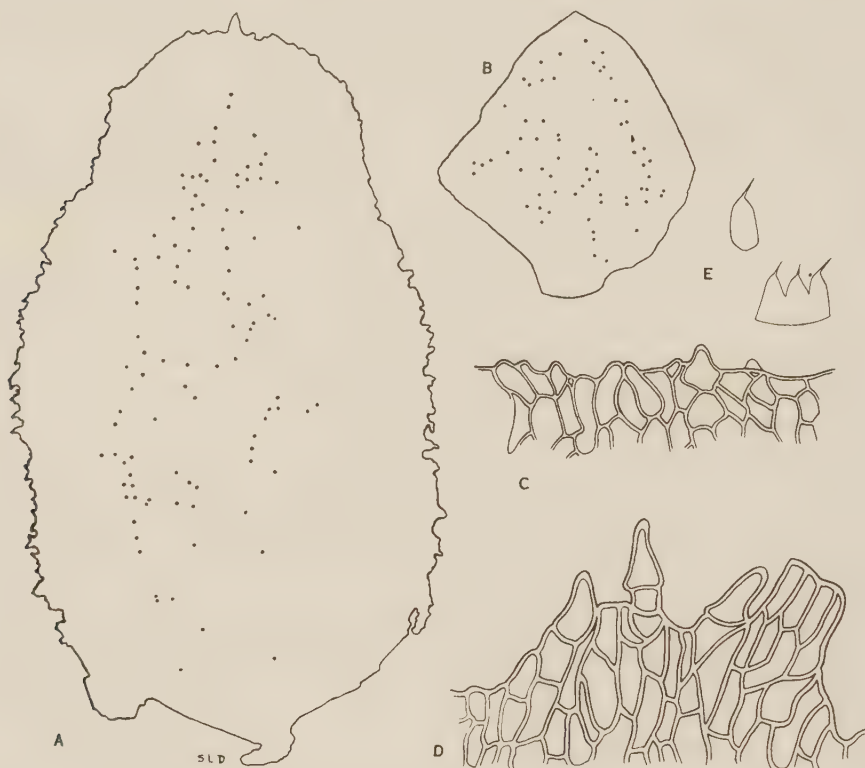


Fig. 3.—Portions of the male cones of recent species of *Araucaria*, section Eutacta.

- A. *A. montana*. Sporophyll, showing the shape, apex, and the distribution of the stomata. x15.
- B. *A. beccarii*. Sporophyll, showing the shape, apex, and the distribution of the stomata. x15.
- C. *A. rulei*. Portion of the sporophyll, showing unicellular, ungrouped projections. x150.
- D. *A. columnaris*. Portion of the sporophyll, showing multicellular, grouped projections. x150.
- E. *A. columnaris*. Sexinous granules of the pollen grain. x10,000.

Recent Species

The recovery from the male cones of *A. lignitici* of large, spherical, non-aperturate, finely granular pollen grains identical with those of the family

Araucariaceae clearly indicates a connection with this family. However, since the pollen grains of *Agathis* and *Araucaria* are indistinguishable from one another, they provide no indication of the generic identity of the cones. It is only when detailed comparisons are made between the cones of *A. lignitici* and those of recent types that a greater resemblance to *Araucaria* becomes apparent.

The male cones of the two sections of *Araucaria* differ from one another both as regards their size and the arrangement of the sporophylls. Cones of the section *Colymbea* range from 7.5 to 17.5 cm. in length and the sporophylls are arranged in verticils (Seward and Ford 1906, p. 318), whereas those of the section *Eutacta* are smaller, ranging from 2.5 to 8 cm. (except *A. muelleri*), and the sporophylls are spirally arranged. In both respects, the agreement of the fossil cones is with those of the section *Eutacta* rather than *Colymbea*. As this similarity is further emphasized by the occurrence of a similar transition from sporophylls to foliage leaves and the detailed structure of the lamina of the sporophyll, the possibility of a connection with the section *Colymbea* will not be considered further.

The male cones of the section *Eutacta* are dense, cylindrical structures consisting of numerous spirally arranged sporophylls. The lamina of the sporophyll is usually relatively small and either rhomboidal or oval to oblong in shape (Fig. 3*b*, 3*a*). The apex may be acute or obtuse; sometimes a minute mucro is present (Fig. 3*a*). The margins are either entire or minutely crenulate, and the marginal cells of all species, except *A. muelleri*, are prolonged into bluntly pointed unicellular or multicellular denticulations of varying length (Fig. 3*c*, 3*d*), which towards the base of the lamina are frequently recurved. Their outer walls are sometimes strongly thickened. The cells of the outer epidermis are usually irregular in shape, but are often elongated in a longitudinal direction. Their lateral walls vary in thickness; they are always pitted and are either curved or sinuous. The stomata, which are moderately numerous, are usually restricted to the central portion of the lamina. Only those of *A. muelleri* and *A. rulei* are arranged in long longitudinal rows.

The cells of the upper epidermis are usually narrower and more elongated than those of the lower surface. Their lateral walls are thin, pitted or unpitted, and straight or sinuous. Stomata are absent, sparsely scattered or, as in *A. muelleri* and *A. rulei*, relatively numerous and arranged in rows.

The large, spherical, non-aperturate form of the pollen grains of the Araucariaceae is well known but no systematic account of the pollen grains of recent species has been given. Measurements of acetolysed pollen grains of the section *Eutacta*, made during the present investigation, have therefore been included in Table 2.

The disagreement existing regarding the sculpture of the exine has necessitated a more detailed examination of this layer. Thibout (1896), using pollen of *Araucaria columnaris*, and Cookson (1947*b*), using pollen of *Araucaria bidwillii* Hook. and *Agathis brownii*, have classed the exine as granular, whereas

Wodehouse (1935), from pollen grains of *Araucaria araucana* (Mol.) K.Koch and *Agathis philippinensis*, and Cranwell (1941), from *Araucaria excelsa*, have concluded that the exine is pitted.

From sections of acetolysed exines of *Araucaria columnaris*, it has been possible to determine that the sexine is composed of numerous closely placed, oval granules (pilae), and that each of these terminates in a short spine (Fig. 3; Plate 2, Fig. 16). The granules are of slightly different sizes (Plate 2, Figs. 17 and 18), but their length approximates fairly closely to 1 μ . As the sexine can be detached from the nexine as a complete layer, it seems probable that the bases of the pilae are loosely connected to one another. Isolated granules have been observed in the preparations.

Comparison of Araucaria lignitici with Recent and Fossil Species

Recent species.—Reference to the previous section and to Table 2 shows that the male cone and pollen grains of *A. lignitici*, while falling within the generalized description of those of living members of the genus *Araucaria*, section Eutacta, cannot be exactly matched with any particular living species. The cone appears to have been smaller than most of these, but the incompleteness of the available samples precludes satisfactory comparison on a size basis.

As regards the size and shape of the lamina of the sporophyll, it can be said that they are so distinct from those of *A. columnaris*, *A. excelsa*, and *A. montana* (Fig. 3) as to make it appear unlikely that the parent of the fossil cone was closely related to any of these species. On these and other grounds, it appears probable that the closest connection is with *A. beccarii*, *A. bernieri*, *A. cunninghamii*, and perhaps with *A. balansae* and *A. biramulata*.

Fossil species.—There have been no previous records of the male cones of *Araucaria* from Tertiary strata in Australia. In fact the only other fossil male cone of araucarian type ever recorded is that of *Brachyphyllum mamillare* Brongn., which was described by Kendall (1949). This species occurred in the mid-Jurassic estuarine series of north Yorkshire. Its general conformation is in agreement with that of *Araucaria lignitici*, but the pollen grains are quite distinct.

(c) *Female Cone-Scales*

Araucaria lignitici

(i) *External Characters* (Plate 3, Figs. 19-21)

The cone-scales are broadly cuneate, with a length of 11-18 mm. and a breadth at the distal end of 9-11 mm. A slightly thickened central area marks the position of the seed, and there are two well-defined lateral wings. The broad distal portion of the scale is prolonged into a flat, narrow spine about 4 mm. long. A broad (about 4 mm.), shallow ligule is inserted on the upper surface of the scale a short distance beyond the distal limit of the seed.

(ii) *Cuticular Structure* (Plate 3, Figs. 22-24)

The epidermal cells, which are essentially similar on both surfaces, vary in size and shape according to their position on the scale.

TABLE
MORPHOLOGICAL AND CUTICULAR CHARACTERS* OF THE MALE CONES

Species	Cone Size (cm.)		Lamina of th									
	Length	Width	Shape Rhomboidal†	Apex Acute	Mucro Present†	Marginal Denticulations				Shape		
						Numerous	Thick-walled	Grouped†	Multicellular†	Irregular	Elongated and Parallel	Thick
<i>A. balansae</i>	5-7.5	1.5-2.5	+	+	+	+	+	-	-	+	-	+
<i>A. beccarii</i>	c. 5	0.5-1	+	-	-	+	-	-	-	+	-	-
<i>A. bernieri</i>	7-9	c. 0.8	+	-	-	+		-	-	+	-	+
<i>A. biramulata</i>	6-7	1.5-2	+	+	+	-	+	-	-		+	×
<i>A. columnaris</i>	2.5-7.5	1-2	-	+	+	+	+	+	+	+	+	-
<i>A. cunninghamii</i>	5-7.5	1-2	+	+	-	+	-	-	-	+	-	-
<i>A. excelsa</i>	5-7.5	c. 2.5	-	-	+	+	-	+	+	+	+	-
<i>A. montana</i>	?	c. 1.5	-	-	+	+	+	+	+	+	+	-
<i>A. muelleri</i>	< 25	< 3.5	+	+	?	-	-	-	-	+, -	+	-
<i>A. rulei</i>	c. 5	c. 2.5	+	+	+	-	+	-	-	+	+	-
<i>A. lignitici</i>	c. 2	c. 0.5	+	+	+	+	-	-	-	+	-	+

* See footnote to Table 1.

† Under 'Shape Rhomboidal' — indicates an oval to oblong shape (Fig. 3a). The mucro referred to is the minute terminal projection present on the lamina of the sporophyll of some species.

ophyll

Epidermis				Upper Epidermis							Pollen Diameter (μ)	
Stomata		Cells Elongated and Parallel	Walls			Stomata			Average	Range		
Pitted	Present		Numerous	In Rows	Thick	Simuous	Pitted	Present			Numerous	In Rows
+	+	+	—	+	—	—	+	—	—	—	70	49-86
+	+	—	—	+	—	×	—	—	—	—	66	44-80
+	+	+	—	+	—	—	+	—	—	—	?	?
+	+	×	+	+	—	—	+	+	—	×	?	?
+	+	—	—	+	—	—	+	+	—	—	74	60-86
+	+	+	—	+	—	—	+	+	—	—	68	57-77
+	+	—	—	+	—	—	+	+	—	—	66	40-88
+	+	+	—	+	—	—	+	+	—	—	?	?
+	+	+	+	+	—	+	+	+	+	+	80	60-93
	+	+	—	+	—	—	+	+	—	+	71	60-81
+	+	—	—, +	+	—	—	+	—	—	—	59	52-68

‡ The marginal projections are regarded as being 'grouped' when the crenulations of the margin are sufficiently large to divide the denticulations into a series of more or less separate groups (Fig. 3*d*). The difference between unicellular and multicellular denticulations is shown in Fig. 3*c* and *d*.

Distal region.—The cells of this area, including those of the terminal spine, are smaller and more regular than those of other regions, being frequently square to quadrangular. A tendency to form rows is evident, especially in and near the spine. Stomata occur on both surfaces. They are arranged in rows that become more clearly defined towards the spine. It is not known how far the stomatal rows extend, as no preparation has shown the complete length of the spine.

Epidermis covering the seed (Plate 3, Fig. 23).—The epidermal cells of this region are always longer than broad ($65\text{--}166 \times 13\text{--}27 \mu$), and those situated towards the periphery are somewhat larger than those of the centre. They are irregularly shaped and variously oriented. Occasionally, groups of similarly oriented cells lie either obliquely or at right angles to neighbouring groups, but the formation of such groups of “sister cells” (Kendall 1949) is not a conspicuous feature of the scales of this species. Stomata are absent from both surfaces.

Wings (Plate 3, Fig. 24).—The epidermal cells are similar to, but somewhat larger than, those covering the seed. Stomata are absent.

Ligule (Plate 3, Fig. 22).—Most of the cells are irregular in shape, but some degree of elongation and parallelism is evident in the centre and at the base near the margins. The cell walls are pitted, straight or curved, and thicker at the apex. Stomata are absent.

Recent Species

The attribution of the fossil cone-scales to a species of *Araucaria* is supported by the presence of an embedded seed, a ligule, and a prominent terminal spine. The section Eutacta is indicated by the lateral wings, the broadly cuneate shape of the scale, and its general cuticular characters.

The external form of the cone-scales of the section Eutacta is very uniform, and this uniformity extends to the cuticle. Cuticular characters that appear to be constant are:

The occurrence of amphicyclic stomata, which tend to form short rows, on both surfaces of the distal region.

The occurrence of epidermal cells oriented around the encircling cells of many of the stomata of the distal region.

The absence of stomata from the upper epidermis covering the seed and ligule.

The presence of smaller and usually more regularly arranged epidermal cells in the distal region.

The narrow-elongate form of the epidermal cells of the proximal region (the type of cell present in three living species is shown in Plate 3, Figs. 25-27).

The more or less frequent occurrence of groups of similarly oriented “sister cells” (Plate 3, Fig. 2*b*).

The presence of small unicellular or multicellular denticulations on the margin of the ligule.

Comparison of Araucaria lignitici with Recent and Fossil Species

Recent species.—It has not been possible to obtain large numbers of specimens of female cone-scales of living species, and consequently the determination of features likely to be of value in separating them has been, in many cases, incomplete. There have also been practical difficulties in the preparation of cuticular mounts of both recent and fossil species.

The cuticular features that appear to vary between the species are set out in Table 3. From these, and from a study of the external characters, it can be seen that the cone-scales of *A. lignitici* do not exactly correspond with those of any of the living species studied. It is possible that the closest resemblance lies with *A. beccarii* and *A. cunninghamii*, but there is not enough evidence available either to confirm this suggestion or to completely exclude other species.

Fossil species.—There are only two previous records of the female reproductive organs of *Araucaria* in Tertiary deposits of the Southern Hemisphere. In 1883, Mueller described the female cone of *A. johnstonii*, which was found as an impression in the travertine of Geilston Bay, Tasmania. As no individual scales were discovered, it is difficult to compare this fossil with scales of *A. lignitici*. However, if the cone of *A. johnstonii* was even approaching maturity, the scales must have been considerably smaller than those of *A. lignitici*. As, in addition, the terminal spine is either absent or differently shaped in the Tasmanian specimen, there appears to be little reason for regarding the two forms as belonging to one species. It is not possible to confirm this view by reference to the leaves, as both Florin (1940) and Selling (1950) agree that the shoot attributed to *A. johnstonii* does not, in fact, belong to *Araucaria*.

Female cone-scales of *Araucarites ruei*, from the Kerguelen Archipelago, were described and illustrated by Seward and Conway (1934). These scales are somewhat similar to those of *Araucaria lignitici*, although the terminal spine is relatively much longer. As mentioned previously, the leaves are different in the two forms, and it seems probable that the cone-scales also belonged to separate species. The great distance of the Kerguelen Archipelago from Victoria also points to the same conclusion.

(d) *Conclusion*

It can be seen from the foregoing section that a complete description is now available of the leaves, male cones, and female cone-scales of a Tertiary conifer, the morphological and anatomical features of which agree with those characteristic of living species of the genus *Araucaria*, section *Eutacta*. While the generic identity of *A. lignitici* is regarded as being proved, its specific affinities remain uncertain. Although its leaves appear to be closest to those of *A. balansae*, they do not greatly differ from those of *A. cunninghamii*. The fact that both the male cones and female cone-scales resemble those of *A. beccarii* and *A. cunninghamii* (which according to Dallimore and Jackson are closely allied) suggests that *A. lignitici* may be related to the recent Australian species *A. cunninghamii*.

TABLE 3
MORPHOLOGICAL AND CUTICULAR CHARACTERS* OF THE FEMALE CONE-SCALES OF
RECENT AND FOSSIL SPECIES OF *ARAUCARIA*, SECTION EUTACTA

Species	External Features		Epidermis										Length of Cells (μ)					
			Terminal Spine					Covering Seed										
			Lower Surface		Upper Surface			Lower Surface		Upper Surface								
			Cell Walls Thick	Cell Walls Sinuous	Cell Walls Pitted	Cell Walls Thick	Cell Walls Sinuous	Cell Walls Pitted	Cell Walls Thick	Cell Walls Sinuous	Cell Walls Pitted	Cell Walls Thick		Cell Walls Sinuous	Cell Walls Pitted	Sister Cells Frequent		
<i>A. balansae</i>	c. 2.5	c. 3	+	—	+	+	—	+	—	+	—	+	—	+	—	+	×	80-390
<i>A. beccarii</i>	c. 2	c. 1.5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	110-650
<i>A. bernieri</i>	c. 3	2.5-2.8	+	—	+	+	—	+	—	+	—	+	—	+	—	+	—	40-580
<i>A. biramulata</i>	c. 3	c. 2.2	+	—	+	+	—	+	—	+	—	+	—	+	—	+	—	50-330
<i>A. columnaris</i>	2.5-4.5	2-4.5	×	+	—	+	×	+	+	+	+	+	+	+	+	+	×	80-490
<i>A. cunninghamii</i>	c. 2	c. 3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	40-330
<i>A. excelsa</i>	2-4	2-3	×	+	+	+	×	+	+	+	+	+	+	+	+	+	+	50-470
<i>A. humboldtensis</i>	c. 2.5	c. 2	+	+	+	+	+	+	+	+	+	×	+	+	+	+	+	110-490
<i>A. muelleri</i>	c. 3	c. 2	+	—	+	+	+	+	+	+	+	+	+	+	+	×	+	140-760
<i>A. rulei</i>	c. 3	c. 2	+	—	+	?	?	?	?	?	?	+	+	+	+	+	+	130-560
<i>A. lignitici</i>	1.1-1.8	0.9-1.1	+	—	+	+	×	+	+	+	+	+	+	+	+	×	+	40-270

* See footnote to Table 1.

† The ligules classed as being longer than broad are not very much longer, and the length and width may even be approximately equal; however, they do appear to be distinct from the remainder, in which the breadth is considerably greater than the length.

‡ The letter *u* in the columns referring to the thickness of the epidermal cell walls indicates that they are thickened unequally (Plate 3, Fig. 27).

§ The sister cells mentioned are discussed in the text.

VI. AGATHIS

Leaves of a species of *Agathis* to be described here, under the name *Agathis yallournensis* were collected from various levels in the Yallourn open cut. They were particularly abundant in the layer from which specimens of *Araucaria lignitici* were obtained. Two female cones of *Agathis* were also found at the latter horizon. Although there is no proof that they belonged to the same species as the leaves, the absence of any evidence indicative of a second species is regarded as sufficient justification for including them in the description of *Agathis yallournensis*.

Fragments of leaves referable to *Agathis* were also collected from a leaf-bed which, in 1947, overlay the brown coal on the north side of the Lucifer Colliery, Bacchus Marsh, Vic. These leaves appear to differ from those of *A. yallournensis* in certain well-defined and apparently constant cuticular features, and there is little doubt that they represent another species. Although the remains are so fragmentary that a complete description of the external form cannot be obtained, the fact that the identification of this species would, even if complete leaves were available, probably depend on cuticular characters, is sufficient reason for naming it specifically. It will be described as *Agathis parwanensis*, the specific name referring to the Parwan valley in which the coal seam lies.

(a) *Leaves**Agathis yallournensis* n. sp.

(= *Agathis intermedia* (Ett.) Chapman and Crespin in part)

(i) *External Characters* (Plate 4, Figs. 28-32)

With the exception of the small example shown in Plate 4, Figure 32, which is only 3.5 cm. long, all the leaves have been incomplete. They are narrow- to broad-lanceolate, with an obtuse apex and a petiolar region about 1.5 mm. wide. The greatest length observed has been 9.5 cm. without the proximal region. The width of the lamina ranges from 1 to 2 cm. The margin is entire, flat, and not appreciably thickened. The veins are numerous, parallel, and frequently inconspicuous.

(ii) *Cuticular Structure* (Plate 4, Figs. 33-36)

Lower epidermis.—The stomata are arranged either irregularly or in short longitudinal rows; their orientation is variable, the polar axis of the majority being oblique (Table 4). They are large, their polar diameters ranging from 52 to 65 μ , and amphicyclic, with from four to six small, thick-walled subsidiary cells, one or two of which are frequently polar, and a corresponding number of encircling cells. The outer stomatal chamber is wide and elliptical, square, or polygonal in shape.

The epidermal cells between the stomata vary considerably both in size and shape. They are either square, quadrangular, or irregular in surface view, with their long axes parallel to the length of the leaf. Their lateral walls are straight or slightly curved, about 2.5 μ thick, and inconspicuously pitted. From

one to several rows of narrow, elongated cells without stomata occur at intervals and extend for some distance in a longitudinal direction. Two narrow zones without stomata also occur at the margins.

Upper epidermis.—The cells are from four- to six-sided, or irregular, with straight, pitted lateral walls approximately $3\ \mu$ thick. They are sometimes arranged in longitudinal rows. A few stomata may be present.

(iii) *Internal Anatomy* (Plate 5, Figs. 37-39)

The internal structure of the leaves of *A. yallournensis* has been studied from sections prepared by the paraffin method. The mesophyll, especially the central zone, is frequently compressed.

The cells of the upper epidermis are from square to quadrangular in transverse section. Their outer walls are very thick ($10.5\ \mu$) and highly cutinized. A cryptocrystalline zone is conspicuous. Beneath the upper epidermis there is a one-layered hypodermis composed of isolated, thick-walled fibres, which are most numerous at the edges of the leaves, and of thinner-walled, more or less square cells. A similar layer, although less strongly developed, is present on the under surface. The lower epidermis is broken at intervals by the stomata. The guard cells are depressed below the surface and over-arched by projections of the subsidiary cells, which in turn are partially covered by the encircling cells. The walls of the guard cells, especially the outer ones, are considerably thickened.

The mesophyll is differentiated into palisade and spongy parenchyma. Secretory canals, which alternate with the veins, run through both tissues. They are sharply delimited by a layer of small, rather thick-walled supporting cells; sometimes the remains of the secretory layer itself are also evident.

Large, thick-walled, branched sclereids occur in the spongy mesophyll and, less frequently, in the palisade parenchyma. Occasionally minute crystals can be detected on the arms of the sclereids. In spite of their thickened walls, the sclereids are frequently compressed, so that it is difficult to estimate their frequency; they appear to have been moderately numerous. A few fibres occur above and below the vascular bundles. Transfusion tracheids have not been distinguished with certainty.

Recent Species

The similarity that exists between the external form of the leaves of *A. yallournensis* and those of recent species of *Agathis* is also shown in the cuticle and the internal tissues, and consequently comprehensive comparisons of the fossil with living species have been limited to this genus. The only other coniferous leaves that resemble those of *Agathis* in external form belong to certain species of *Podocarpus*, but according to Florin (1931, 1951) they differ from *Agathis* in having longitudinally oriented stomata and in other cuticular features. A study of 14 recent species of *Agathis* has been made, and the resulting details, together with relevant information from Bertrand (1874), Thomson (1905), Seward and Ford (1906), Florin (1931), and Dallimore and Jackson (1948), are recorded in Table 4.

The following features, which can all be compared with those of the fossil leaves of *Agathis* described in this paper, are common to the leaves of the recent species studied:

They are flat, moderately large, and have numerous fine, parallel veins.

The stomata are amphicyclic. The sunken guard cells are overarched by from four to six subsidiary cells, which are themselves partly covered by a corresponding number of encircling cells. The stomata occur chiefly on the lower surface, but a few may also be present on the upper surface. They are arranged in more or less regular longitudinal rows, separated by bands of elongated epidermal cells that may overlies the veins or be irregularly placed, and are obliquely, transversely, or longitudinally oriented.

The epidermal cells have more or less straight, pitted lateral walls. The outer walls of the epidermal cells are from 10 to 18 μ thick, and heavily cutinized; they contain more or less closely contiguous layers of minute crystals of calcium oxalate.

A hypodermis composed of irregularly shaped, thin-walled cells and a varying proportion of fibres is present on both surfaces, but is less strongly developed on the lower one.

The mesophyll is differentiated into palisade and spongy tissues. Thick-walled, branched sclereids occur in the mesophyll. Their arms are studded with crystals of calcium oxalate.

Secretory canals, which alternate with the vascular bundles, are present in the mesophyll. The vascular bundles are accompanied by thick-walled fibres.

Comparison of Agathis yallournensis with Recent and Fossil Species

Recent species.—It is difficult to compare the leaves of *A. yallournensis* with those of any particular living species because of the great similarity in external form throughout the genus, and the fact that the minor variations of internal and cuticular structure are of uncertain constancy within a species. The leaves of *A. yallournensis* were apparently longer than those of *A. australis*, *A. celebica*, *A. flavescens*, and *A. philippinensis*, but the variations in shape between the living species are of little value for comparative purposes.

There is little difference in the general appearance of the cuticle of the recent species, and the stomata are of almost uniform size, the average polar diameter of the guard cells ranging only from 44 to 56 μ . However, the orientation of the stomata may prove to be of diagnostic value, as on this basis it appears to be possible to divide the species into the two following groups:

(A) Species in which 30 per cent. or more of the stomata are placed transversely.* The number of longitudinal stomata is very small—5 per cent. or less, and frequently only 1 or 2 per cent.—and the number of oblique ones is less than 70 per cent.

(B) Species in which less than 30 per cent. of the stomata are transversely placed. The number of longitudinal stomata is variable, but tends to be

* In calculating the percentage of transversely and longitudinally oriented stomata, only those exactly at right angles to, or parallel with, the axis of the leaf are regarded as being transverse or longitudinal; all others are classed as oblique.

greater than in group (A), and may be up to 30 per cent. Oblique stomata are usually more frequent than in group (A), and may be up to 91 per cent.

Examination of Table 4, which gives the percentages of variously oriented stomata in all the living species examined, shows that there is a gradation between species such as *A. celebica*, which have a very high proportion of transversely placed stomata, and those such as *A. lanceolata*, with a very low one. The percentage (30) taken as the dividing line between groups (A) and (B), is therefore purely arbitrary, and in some cases it is even difficult to determine, with certainty, the group into which a given species should be placed. However, those species with either very low or very high proportions of transversely placed stomata do appear to be clearly and constantly distinct.

Agathis yallournensis belongs to group (B) and is thus similar to *A. australis*, *A. lanceolata*, *A. moorei*, *A. palmerstoni*, and *A. philippinensis* as regards stomatal orientation. As the leaves of *A. australis* and *A. philippinensis* are smaller than those of *A. yallournensis*, the fossil leaves appear to be more like those of *A. lanceolata*, *A. moorei*, and *A. palmerstoni*. A consideration of the characters set out in Table 4 suggests that, of these three species, *A. palmerstoni* is the one most similar to *A. yallournensis*. However, this suggestion is only tentative, although the presence of *A. palmerstoni* in Australia at the present time makes this view not unreasonable.

Fossil species.—The only Australian Tertiary fossil leaves previously referred to *Agathis* are those of *A. intermedia* (Ett.) Chapman and Crespin, and *Dammara podozamioides* Ett., now to be called *Agathis podozamioides*. These leaves were first discovered as impressions in Tertiary mudstones from Vegetable Creek, New South Wales, and were described by Ettingshausen (1888). Deane (1925) attributed to *A. intermedia* certain leaf fragments from the Morwell brown coal mine, now known as the Yallourn North open cut, and Chapman and Crespin (1934) recorded *A. cf. intermedia* from Miocene beds at Cape Riche, Western Australia.

As far as external features are concerned, the leaves of *A. yallournensis* appear to be somewhat different from both *A. intermedia* and *A. podozamioides* as they were originally described by Ettingshausen. The petiole of *A. podozamioides* is considerably wider than those of the leaves from Victoria, and the leaves of *A. intermedia* appear to be relatively wider and to taper more sharply towards the apex. Perhaps the closer resemblance lies with *A. intermedia*, but the differences that exist, and the fact that no information is available regarding the leaf structure of that species, appear to justify the creation of a new species.

The leaves referred by Deane to *A. intermedia* are regarded as being identical with those of *A. yallournensis*. Fragments of cuticle removed from one of Deane's (1925, Fig. 12) specimens agree in all features with the cuticles from *A. yallournensis*.

The record of *Agathis* by Chapman and Crespin referred to above, is open to considerable doubt. The specimen on which these authors based their

determination has been available for re-examination, and, since it was not figured by them, is illustrated in Plate 5, Figure 44. The fossil is only a portion of a larger impression that apparently lay obliquely in the stone. Owing to its fragmentary nature, only the left-hand margin is intact, and a true estimate of its original shape cannot be formed. The surface of the impression is covered by a light brown film of mineral, and is distinctly marked by regularly arranged, longitudinal ridges separated by a corresponding number of narrow grooves, of which there are 12 in the widest part. While Chapman and Crespin's identification as *Agathis* cf. *intermedia* may be partially or wholly correct, the specimen does not seem to be adequate for a generic determination. Much clearer evidence than it provides is necessary before *Agathis* can be regarded as a component of the Tertiary flora of Western Australia. It is not possible to compare this very incomplete specimen with *A. yallournensis*.

Agathis parwanensis n. sp.

(i) *External Characters* (Plate 6, Fig. 45)

The example figured is the best of a small collection of this type. The leaf fragment is 2.8 cm. long, and in that length widens gradually from 0.4 to 0.9 cm. Its surface is marked by 12 parallel veins, and its margin is entire. As none of the remaining specimens exceeds 1 cm. in width, it is probable that the leaf of this species was a rather narrow one.

(ii) *Cuticular Structure* (Plate 6, Figs. 46, 47)

Lower epidermis.—The leaves are amphistomatic. The stomata are rather regularly arranged in longitudinal rows that extend for varying distances, the rows being separated by longitudinally elongated epidermal cells. Associated with the stomata are four, rarely five or six, subsidiary cells, two of which are frequently polar, and an equal number of encircling cells. Usually each stomatal group has its full complement of encircling cells, but sometimes two groups have an encircling cell in common, and in a few instances subsidiary cells of adjacent groups have been observed to abut on one another. The average polar diameter of the guard cells is $50\ \mu$ and the range from 44 to $55\ \mu$. Their orientation is variable, but as many as 50 per cent. have been observed to be transversely oriented.

The epidermal cells are square, quadrangular, or somewhat irregular in shape, the long axis frequently lying in a longitudinal direction. The lateral walls are straight or curved, about $2.5\ \mu$ thick, and rather strongly pitted.

Upper epidermis.—The epidermal cells, which are usually square to quadrangular in shape, tend to be arranged in longitudinal rows. Their lateral walls are straight, about $2.5\ \mu$ thick, and fairly strongly pitted. Stomata have not been observed.

(iii) *Internal Anatomy*

It has not been possible to prepare very satisfactory sections of the leaves of *A. parwanensis*, and consequently the following description of the internal

Species	External Features				Internal Structure					
	Size (cm.)		Shape Lanceolate†	Apex Acute	Sclereids			S.C.‡		Fibres Numerous§
	Length	Width			Numerous	In Palisade	In Sp.Mes.	In Palisade	In Sp.Mes.	
<i>A. alba</i> (Rumph.) Warb.	5-12.5	1-5	±	±	+	+	+	—	+	—
<i>A. australis</i> Salisb.	1.5-5	1-2	—	—	+	—	+	—	+	+
<i>A. brownii</i> (Lemaire) L. H. Bail.	5-15	2.5-6	±	±	×	+	+	—	+	×
<i>A. celebica</i> Warb.	c. 4.5	c. 2.5	+ —	—	—	+	+	—	+	+
<i>A. flavescens</i> Ridl.	5-6.5	1-2.5	—	—	+	+	+	+		×
<i>A. lanceolata</i> (Panch.) Warb.	4-12.5	0.5-1.5	±	+	—	+	—	—	+	—
<i>A. microstachya</i> J. F. Bailey and C. T. White	3-5	1.2	+	—	×	—	+	+	+	—
<i>A. moorei</i> (Lindl.) Mast.	6-13	1-4	±	—	—	+	—	—	+	
<i>A. obtusa</i> (Lindl.) Morrison	12	3	—	—	+	+	+	—	+	—
<i>A. ovata</i> Warb.	7.5-11.5	1.5-3	—	—	+	+	+	+	+	—
<i>A. palmerstoni</i> F.v.M.	5-10	1-2	±	—	+	+	+	—	+	+
<i>A. philippinensis</i> Warb.	7.5	1-2.5	+	±	—	—	+	+	+	+
<i>A. regia</i> Warb.	7.5-9	c. 3	+	—	—	—	+	+	+	+
<i>A. vitiensis</i> Benth. and Hook.	5-15	0.5-4.5	—	—	—	—	+	—	+	+
<i>A. parwanensis</i>	?	?	?	?	—	?	+	+	+	+
<i>A. yallournensis</i>	c. 9.5	1-2	+	—	—	+		+	+	+

* See footnote to Table 1.

† In deciding whether a leaf should be classed as lanceolate, all variations

‡ S.C. = secretory canals; Sp.Mes. = spongy mesophyll.

§ The fibres referred to in the table are those lying immediately beneath

** In all species, at least some of the stomata are in rows, but they are counted after the percentage orientation of each species is that of averages taken from the group. The group refers to the distribution of the variously oriented stomata as described.

†† The cells of the upper epidermis always show some degree of regularity, the cells almost uniformly elongated in a direction parallel with that of the axis of junctions of the lateral walls of the epidermal cells.

Cuticle										
Surface					Upper Surface Epidermal Cells††					
Stomata**					Walls					
Range	Transverse (%)	Oblique (%)	Longitudinal (%)	Group	In Rows	Elongated and Parallel	Thick	Sinuous	Pitted	Thickened Corners
Stoma Diameter (μ)										
39-57	44(30-52)	55(47-69)	1(0-2)	A	+	—	—	—	+	—
48-53	13(4-19)	78(73-85)	9(4-11)	B	+	—	—	×	+	—
44-57	34(30-38)	63(60-67)	3(2-5)	A	×	—	+	—	+	—
47-57	49(42-55)	50(44-56)	1(1-2)	A	—	—	—	—	+	+
45-53	40(33-48)	58(50-65)	2(1-3)	A	—	—	+	—	+	+
49-60	7(2-9)	80(78-91)	13(7-17)	B	—	—	+	—	+	+
55-62	15(12-17)	84(82-85)	1(1-3)	B	—	—	+	—	+	—
44-52	20(15-26)	76(69-82)	4(2-5)	B	+	+	+	—	+	—
40-51	43(33-55)	57(44-67)	0(0-1)	A	+	+	—	—	+	—
51-59	17(8-28)	70(67-84)	13(1-24)	B	—	—	+	—	+	—
47-57	12(7-19)	75(64-82)	13(5-29)	B	—	—	+	—	+	—
44-49	18(13-24)	75(68-80)	7(4-9)	B	—	—	+	—	+	—
53-61	33(30-38)	64(61-66)	3(1-4)	A	—	—	+	—	+	—
45-53	34(32-36)	64(63-65)	2(1-3)	A	—	—	+	—	+	—
44-55	41(32-50)	56(48-62)	3(2-6)	A	+	+	—	—	+	—
52-65	8(3-16)	79(67-88)	13(6-24)	B	—	—	—	—	+	—

long-lanceolate, etc., have been included in this term.

majority are in long, clearly defined rows. The range given in brackets (about 500 stomata) on different leaves and on different parts of one leaf.

It is only in a few species that the rows are long and clearly defined and the term 'thickened' refers to a thickening which is sometimes present at the

anatomy may require modification if further material is obtained. The state of preservation of the internal tissues is not good, but sufficiently so to indicate structural affinity with leaves of recent species of *Agathis*.

The cells of the upper epidermis are rectangular in cross section, with outer walls from 9 to 11 μ thick. A cryptocrystalline zone is present beneath the cuticle. The hypodermis is composed chiefly of fibres, which form an almost continuous layer. The cells of the lower epidermis are similar to those of the upper surface, but the outer walls are thinner, being only from 4.5 to 5.5 μ thick. Isolated fibres lie beneath the lower epidermis. It has not been possible to observe the exact nature of the subsidiary and encircling cells of the stomatal apparatus, but the guard cells can be seen to be thick-walled and deeply sunken. The outer walls are particularly thick.

The mesophyll is differentiated into spongy parenchyma and a palisade layer that is two cells thick. Secretory canals are present, but they appear to have become partially disintegrated and, unless some contents are preserved, are considerably compressed. They occur in both the palisade and spongy parenchyma. A few indistinct cell remains have been interpreted as sclereids.

The vascular tissues are very much compressed, and hence the details of their structure are obscure. Fibres may have been associated with the bundles, but they cannot be recognized with certainty.

Comparison of Agathis parwanensis with Recent and Fossil Species

Recent species.—The incompleteness of the specimens available precludes satisfactory comparison with recent species on the basis of their external features.

In having a stomatal orientation of group (A), leaves of *A. parwanensis* resemble those of *A. alba*, *A. brownii*, *A. celebica*, *A. flavescens*, *A. obtusa*, *A. regia*, and *A. vitiensis*. When other cuticular and anatomical features are taken into consideration, the closer agreement between *A. parwanensis*, *A. alba*, and *A. brownii* becomes evident. However, the meaning of this apparent resemblance is rather uncertain, particularly as the leaves of both living species are usually much wider than any so far recorded for *A. parwanensis*. It is perhaps of interest to note in this connection that *A. brownii* occurs in southern Queensland.

Fossil species.—It is impossible to compare *A. parwanensis* with either of the previously recorded Australian Tertiary species of *Agathis*, as the descriptions of these were based on external form alone.

A. parwanensis differs from *A. yallournensis* as regards stomatal orientation, stomatal size, the arrangement of the epidermal cells of both surfaces, and the relative abundance of hypodermal fibres (Table 4). There seems to be little doubt that the leaves from Bacchus Marsh and Yallourn represent two distinct species, but it is unfortunate that the complete leaves and male and female cones of *A. parwanensis* are not available so that the question could be settled with certainty.

*(b) Male Cones**Agathis yallournensis*

The specimen illustrated in Plate 5, Fig. 40, is the only one of its kind in the present collection. It was discovered by Mr. H. A. Adams on June 4, 1950, in a sample of leafy coal taken from the Yallourn open cut at a locality the coordinates of which he estimated to be 1900E. and 6800S. and the reduced level 60.

Pollen grains recovered from this fossil show conclusively that it is a male cone of a member of the family Araucariaceae, but do not indicate whether it belonged to a species of *Agathis* or *Araucaria*. Agreement regarding the external form of the cone and the microscopic features of the laminae of the sporophylls, as well as the smaller size of its pollen grains, however, suggest a connection with *Agathis* rather than *Araucaria*. The occurrence of leaf fragments of *Agathis yallournensis* on the same coal sample is additional evidence supporting such a relationship. As *A. yallournensis* is the only representative of the genus as yet recognized at Yallourn, it seems reasonable that the cone under consideration should be specifically associated with the leaves of this type.

(i) External Characters (Plate 5, Fig. 40)

The specimen consists of a short length of a woody axis 3 mm. wide and a terminal cone. The cone is cylindrical, with an obtuse apex, and is 0.8 cm. broad at its widest part and 3.2 cm. long. It contains a large number of small sporophylls arranged in a close spiral. The laminae of the sporophylls are small, approximately 1.5×1.3 mm., and are thick and roughly oval in shape, those towards the base being slightly larger and more hemispherical. Bracts are not indicated.

(ii) Cuticular Structure of the Lamina of the Sporophyll (Plate 5, Fig. 41)

Lower epidermis.—The epidermal cells are irregular in shape and arrangement, and have straight or curved, inconspicuously pitted lateral walls about 2.5μ thick. Stomata of araucarian type are relatively numerous and are scattered over the whole surface.

Upper epidermis.—The cells tend to be elongated in a longitudinal direction and to be arranged in rows. The cell walls are thinner than those of the lower surface, straight or curved, and unpitted. Stomata occur infrequently.

Margin.—The margin is even, but many of the marginal cells are produced into blunt, thin-walled denticulations.

(iii) Pollen Grains (Plate 5, Figs. 42, 43)

Pollen grains have been recovered in abundance from the cone of *A. yallournensis*. They are usually flattened and frequently crumpled and fragmentary, but in favourable examples the spherical form is discernible. The pollen grains are non-aperturate and very variable in size. The average diameter of 72 acetolysed grains was 46μ and their range from 34 to 62μ . The exine is

two-layered and approximately $2\ \mu$ thick. The sexine is composed of small, rather closely arranged granules, each of which terminates in a minute spine.

Recent Species

In some instances, it is rather difficult to distinguish between the male cones of recent species of *Agathis* and those of *Araucaria*. The cones of *Agathis* are usually smaller than those of *Araucaria*, but the measurements of a few species may overlap. However, the laminae of the sporophylls of the two genera appear to be morphologically and structurally dissimilar in some respects. The laminae of *Agathis* are oval to hemispherical in shape and the cells of the lower epidermis are irregularly arranged; those of *Araucaria*, on the other hand, are more frequently rhomboidal, with acute apices, and the cells of the lower epidermis are somewhat elongated and regularly arranged. It has been the recognition of similar differences in the fossil cones from Yallourn that has led to the reference of one of them to *Agathis* and the remainder to *Araucaria*.

In other respects, both the external form of the male cone and the structure of the lamina of the sporophyll of *Agathis* are similar to those of *Araucaria*, which were discussed earlier in this paper.

The pollen grains of six species of *Agathis* have been examined. All conform to the type described for *Araucaria* (see Section V(b) (iv)) but the average diameters have been consistently smaller. They range from 40 to 56 μ , whereas those of *Araucaria* range from 59 to 81 μ .

Comparison of Agathis yallournensis with Recent and Fossil Species

Recent species.—The male cone of *A. yallournensis* agrees with the general morphological and structural features of those of the genus *Agathis*, but does not appear to be identical with the cones of any of the living species studied.

As shown in Table 5, the size and shape of the cone and of the lamina of the sporophyll of *A. yallournensis* resemble those of *A. brownii* and *A. palmerstoni* most closely, and this resemblance is also evident in the cuticular features of the lamina.

Fossil species.—There are no previous records of the male cones of Tertiary species of *Agathis* in Australia.

(c) *Female Cones*

Agathis yallournensis

The two specimens of the female cone, here associated with *Agathis yallournensis*, were found at the third level of the Yallourn open cut in the bed that included remains of both *Araucaria lignitici* and *Agathis yallournensis*. The general features of the cone and the occurrence of stomata of araucarian type in the epidermis of the cone-scales suggest that it belonged to a species of the family Araucariaceae. More detailed comparisons of both macroscopic and cuticular characters indicate that its affinity is with *Agathis* rather than with *Araucaria*.

(i) *External Characters* (Plate 6, Figs. 48-52)

Both cones appear to be immature and to be laterally compressed. Each has a roughly circular outline and it seems probable that the original cones had a spherical form. The smaller of the two, shown in Plate 6, Figures 48-51, measures 1.6 cm. across, the larger 2×2 cm. Both cones are alike, but since the smaller one is more favourably exposed it has been selected as the type specimen.

The cone is composed of a number of individual cone-scales which are spirally inserted on a rather slender axis (2 mm. wide) and imbricately arranged. In the portion of the specimen that is transversely exposed and can be viewed from beneath, the outlines of several entire sporophylls are evident (Plate 6, Fig. 50). Each consists of a broad, sub-acute, somewhat reflexed distal expansion and a proximal portion with straight sides, which narrow considerably towards the axis of the cone. The cone-scales are approximately 7 mm. long and in their widest part 7-9 mm. across. Because of the unsatisfactory state of preservation of the proximal seed-bearing portion of the scale it has not been possible to remove a complete scale for the description of its upper surface. The distal margin of the cone-scale is crenulate. The under surface of the distal expansion is finely ridged.

(ii) *Cuticular Structure of Distal Portion of a Cone-Scale* (Plate 6, Figs. 53, 54)

Lower epidermis.—The cells are square, quadrangular, or irregular, with the long axis parallel to the length of the scale, and tend to be arranged in rows. The cell walls are about $3\text{--}4\ \mu$ thick and very strongly pitted. Stomata are numerous, becoming sparser towards the distal margin.

Upper epidermis.—The cells are larger than those of the lower epidermis. They are mostly quadrangular, with the long axis parallel to the length of the scale, and rather regularly arranged. Their lateral walls are thin (about $2.5\ \mu$) and very strongly pitted. Stomata are absent.

Margin.—Most of the cells at the distal margin of the cone-scale are prolonged into blunt, unicellular denticulations similar to those present in the male sporophylls of both *Agathis* and *Araucaria*.

Recent Species

The female cones of members of the genus *Agathis* are roughly spherical or ellipsoidal in shape and are composed of numerous spirally arranged, imbricate cone-scales. These scales, the broad, thick, distal regions of which are reflexed in relation to the proximal portions, are without the lateral wings, terminal spine, ligule, and embedded seed characteristic of *Araucaria*, section *Eutacta*.

It is not possible to consider the cuticular features of the cone-scale as a whole, because the epidermis of the proximal portion is so lightly cutinized that it is destroyed by "macerating" fluids. However, this portion of the scale is not important for present comparative purposes since the same area is, probably for a similar reason, badly preserved in the cone of *Agathis yal-lournensis*.

TABLE
MORPHOLOGICAL AND CUTICULAR CHARACTERS
FOSSIL SPECIES C

Species	Cone				Margin Denticulations	
	Length (cm.)	Width (cm.)	Length (mm.)	Width (mm.)	Numerous	Long
<i>A. alba</i>	5.0-7.5	1.5-2.5	4-6	5-7	+	+
<i>A. australis</i>	2.5-4.0	0.5-1.5	c. 2.5	2-3	—	—
<i>A. brownii</i>	2.5-10	c. 0.8	c. 1.5	c. 1	+	—
<i>A. lanceolata</i>	c. 2.5	c. 0.8	1-1.5	1-1.5	+	—
<i>A. microstachya</i> ‡	0.4-0.6	?	0.7-1.0	c. 1.0	—	—
<i>A. moorei</i>	c. 1.5**	c. 0.8**	1.5-2.5	1.5-2.0	+	++
<i>A. obtusa</i>	c. 2	c. 1	c. 2	c. 2	×	+,—
<i>A. ovata</i>	2.5-6.5	1-2	2.5-3.5	2.5-4.0	×	—
<i>A. palmerstoni</i>	2-5.5	1.0	1-1.5	1-1.5	—	—
<i>A. philippinensis</i>	2.5-4.0		1-1.5	c. 2	+	
<i>A. vitiensis</i>	2.5-3.0	c. 1	1-2	1-1.5	—	×
<i>A. yallournensis</i>	c. 3.2	c. 0.8	1-2	1-2	+	—

* See footnote to Table 1.

† The figures for the length and breadth of the lamina of the sporophyll have been taken from the most prominent ones mentioned in *Araucaria*.

‡ The measurements of pollen grains have been taken from acetolysed material.

§ According to Mr. Lindsay Smith of Brisbane, the length of the male cones of *A. m.* cones of this species kindly forwarded by him for examination are 2 cm. long and 0.6 cm. wide.

** Cones from Sydney, details of which are recorded here, were much larger.

Lamina of the Sporophyll†												Pollen‡	
Laminal data-												Average Diameter (μ)	Range (μ)
	Lower Epidermis						Upper Epidermis						
	Thick-walled	Mucro Present	Cell Walls			Stomata		Cell Walls			Stomata Present		
			Sinuous	Thick	Pitted	Present	Numerous	Sinuous	Thick	Pitted.			
—	—	—	+	+	+	—	—	+	+	—	54	52-57	
+	+	—	—	+	+	—	—	+	—	—	55	44-60	
+	—	—	—	+	+	—	—	—	—	—	50	47-52	
+	—	—	+	—	+	×	—	—	—	—	44	39-49	
+	—	—	+	+	+	—	—	—	+	—	50	36-60	
+	+	—	—	+	+	×	—	—	+	—	46	39-50	
+	—	—	+	+	+	×	—	×	+	—	?	?	
+	—	+,—	+	+	+	—	—	—	+	—	?	?	
+	—	—	—	+	+	+	—	—	—	—	44	34-49	
—	—	—	+	+	+	×	—	—	+	+	?	?	
+	—	—	—	+	+	×	—	—	+	—	47	39-55	
—	—	—	—	+	+	+	—	—	—	+	46	34-62	

n from measurements of herbarium specimens. The mucro refers to the same terminal

crostachya considerably exceeds the figures given by Bailey and White (1916). Mature de.

The cuticle removed from the distal region of the cone-scales of the species examined (*A. australis*, *A. brownii*, *A. lanceolata*, *A. moorei*, *A. ovata*, *A. palmerstoni*, *A. philippinensis*, and *A. vitiensis*) is broadly triangular in shape; owing to an unequal degree of cutinization the portion of it derived from the lower surface is always longer than that from the upper surface. The margin is minutely crenulate or, in immature scales at least, has some of its cells produced into small, unicellular denticulations similar to those observed in the fossil cone-scales. The cells of the lower epidermis are irregularly arranged and have thick, straight or curved, unpitted or inconspicuously pitted walls. Numerous stomata are present.

The structure of the cuticle of the upper surface is rather similar to that of the lower surface but the stomata are either sparse or absent and the cells are usually more regularly arranged and have thinner walls.

Comparison of Agathis yallournensis with Recent and Fossil Species

Recent species.—The female cones of *A. yallournensis* cannot be reliably compared with those of any particular living species. Their compressed condition and probable immaturity precludes any comparison on the basis of size and shape. They are distinct from cones of all the living species listed above in the conspicuous pitting that characterizes the lateral walls of their epidermal cells.

Fossil species.—Female cones of *Agathis* have not previously been recorded from rocks in the Southern Hemisphere. Impressions of isolated cone-scales of *Agathis* type were mentioned by Ettingshausen (1888, Plate VIII, Fig. 36) and associated by him with leaves of *Agathis intermedia*. The example figured appears to represent a mature cone-scale and is thus not comparable with the cones of *A. yallournensis*.

(d) Conclusion

A reasonably complete description of an undoubted member of the genus *Agathis* has been obtained from leaves and cones found at Yallourn, Victoria. Because the leaves and male cones of *A. yallournensis* both resemble those of the living Australian species *A. palmerstoni* and the female cone, as far as is known, is not dissimilar, it seems possible that the affinities of the fossil lie with this species.

Leaves of a second species, *Agathis parwanensis*, have been distinguished in a deposit near Bacchus Marsh, Vic. The specimens of this type, however, are too incomplete to allow its closer relationships to be determined.

VII. FOSSIL POLLEN GRAINS OF THE ARAUCARIACEAE

Large spherical, non-aperturate pollen grains with granular exines have been recovered in small numbers from brown coal, ligneous clays, and mudstones in south-eastern Australia and Tasmania (Fig. 1). These agree in all respects with the pollen grains of recent species of *Araucaria* and *Agathis* and

the fossil pollen grains of *Araucaria lignitici* and *Agathis yallournensis*. They are also indistinguishable from the pollen grains preserved in the ligneous deposit at the Kerguelen Archipelago, upon which the sporomorph *Araucariacites australis* Cookson (1947b) was based.

As was mentioned earlier, the pollen grains of *Agathis* are generally smaller than those of *Araucaria*, but the fact that the maximum size in *Agathis* may overlap the minimum size in *Araucaria* precludes the reliable separation of isolated pollen grains on this basis. The pollen grains recovered from the Australian deposits are therefore referred to the sporomorph *Araucariacites australis*, without implying more than a family connection with those from the Kerguelen deposits.

TABLE 6
DIAMETERS OF *ARAUCARIACITES AUSTRALIS* FROM AUSTRALIAN DEPOSITS

Location			Range in Diameter (μ)
N.S.W.			
Vegetable Creek.	Eh.192.	Aust. Mus.	34-86
Bingera.	Aust. Mus.		40-73
Vic.			
Yallourn.	'Pollen' coal, 3rd Level, Open Cut		60-80
Berwick.	Nat. Mus.	Vic.	52-83
Lal Lal.	Lig. Clay	Bore 55. 238 ft.	44-50
		Bore 51. 398 ft.	37-59
		Bore 60. 189 ft.	35-57
Anglesea			45-65
Hamilton			47-65
S.A.			
Cootabarlow.	Bore 1.	493-515 ft.	52-86
	Bore 1.	537-538 ft.	43-53
Tas.			
Ouse			52-68

Table 6 gives the location of the deposits at which pollen grains of araucarian type have been found, and records the sizes observed.

VIII. DISTRIBUTION OF *ARAUCARIA* (SECTION *EUTACTA*) AND *AGATHIS* IN AUSTRALIA AND TASMANIA

(a) *Present Distribution*

(i) *Araucaria* (see McArthur 1949).—*Araucaria cunninghamii* is the only representative of the section *Eutacta* still existing in Australia. This species extends from the moist mountain forests of New Guinea, where the annual rainfall is in the vicinity of 142 in. and the average temperature for the coldest month 64°F., to the coastal rain forests of Queensland and north-eastern New South Wales. In the latter state *A. cunninghamii* has been recorded from a number of localities on the Northern Tableland (Baker and Smith 1910). The

most southerly of these is Nambucca Heads, at a latitude of $30^{\circ} 36'S.$, where the average rainfall is 51.26 in. and the average temperature of the coldest month $54.5^{\circ}F.$ The most westerly record in this area for *A. cunninghamii* is near Tenterfield ($152^{\circ} 1'E.$) where the average rainfall is 31.78 in. and the average temperature of the coldest month is $54.2^{\circ}F.$

Araucaria excelsa is the only other member of the section that is a native of a subtropical area. At Norfolk Island, however, in spite of its latitude ($29^{\circ} 3'S.$, $167^{\circ} 56'E.$), tropical conditions prevail; the rainfall is high (53.27 in.), and the average temperature of the coldest month is $60.9^{\circ}F.$

All the remaining species are tropical.

(ii) *Agathis*.—Of the two Australian species, *A. brownii* has a restricted distribution in the coastal scrub of southern Queensland (Francis 1929). Its range is from Tewantin ($20^{\circ} 32'S.$), where the annual rainfall is 67.09 in. and the mean temperature of the coldest month $58.8^{\circ}F.$, to within 60 miles of Maryborough ($25^{\circ} 32'S.$), where the rainfall is 46.05 in. and the average temperature of the coldest month $59.3^{\circ}F.$

Agathis palmerstoni, the northern species, is, according to Swain (1928) "common on the highlands of the Cairns-Ravenshoe tableland at 2,000 to 3,000 feet elevation." In this area the mean temperature of the coldest month is $59.5^{\circ}F.$ and the annual rainfall 65.69 in. Swain further remarks that "The Kauri Pines are trees of the regions of 50 to 80 in. annual average rainfall having little or no seasonal drought. The mean temperature of their habitat varies from 59 to 65 deg. F."

The southern limit of the New Zealand kauri, *Agathis australis*, is near Auckland ($36^{\circ} 51'S.$) where the annual rainfall is 43.95 in. and the average temperature for the coldest month is $51.7^{\circ}F.$

All the remaining species are tropical.

It is evident from these data that both *Araucaria* and *Agathis* flourish in regions with a relatively high degree of humidity and with at least a moderately warm temperature.

(b) Tertiary Distribution

The Tertiary records indicate that both genera extended further south than they do at present.

(i) *Araucaria*, Section Eutacta.—The occurrence of shoots of *Araucaria derwentensis* and a female cone of *A. johnstonii* near Hobart shows that the section Eutacta was represented at least as far south as $45^{\circ} 43'$. It has not, as yet, been recorded further north than Uralla ($30^{\circ} 36'S.$), the area from which Selling (1950) described *Araucaria fletcheri*. In Victoria, two distinct species have been distinguished, *A. balcombensis*, a large-leaved species from Balcombe Bay, near Melbourne, and *A. lignitici*, the smaller and narrower-leaved species from Yallourn.

(ii) *Agathis*.—The only valid Tertiary records of *Agathis* are *A. intermedia* from beds near Vegetable Creek in north-eastern New South Wales (lat. 29° 24'S.), *A. yallournensis* from Yallourn, and *A. parwanensis* from Bacchus Marsh, both situated in Victoria at approximately 38°S. latitude. The uncertainty regarding the record of *Agathis* from Western Australia has already been referred to.

(iii) *Araucariaceae*.—The recognition of pollen grains of araucarian type in ligneous clays at Cootabarlow, near Lake Frome in South Australia (140°E.), has established a wider westerly limit for the family than now exists. At present neither *Agathis* nor *Araucaria* occur much more than 100 miles from the east coast, whereas on the evidence of pollen grains, over 700 miles must have separated the occurrences of the *Araucariaceae* in north-eastern New South Wales from those at Cootabarlow.

The recovery of araucarian pollen grains from ligneous clay at Hamilton extends the time range for the family in Victoria to the Early Pliocene period.

The inclusion of *Agathis* and *Araucaria* in the Tertiary flora of south-eastern Australia and Tasmania, as well as the frequent presence on their leaves of the epiphyllous fungus *Asterothyrites ostiolatus* Cookson (1947a), is compatible with the view, expressed by palaeontologists and palaeobotanists, that the climate during this period was warmer and more humid than it is at present.

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X. REFERENCES

- BAILEY, J. F., and WHITE, C. T. (1916).—Dep. Agric. Stock, Brisbane, Botany Bull. No. 18. 16 pp.
- BAKER, R. T., and SMITH, H. G. (1910).—"A Research on the Pines of Australia." (Dep. Publ. Instruction, N.S.W. Tech. Education Ser. No. 16, 452 pp.)
- BERTRAND, C. (1874).—*Ann. Sci. Nat.* **5**: 20.
- BUCHHOLZ, J. T. (1949).—*Bull. Mus. Hist. Nat. Paris* (2) **21**: 279.
- CHAPMAN, F., and CRESPIN, I. (1934).—*J. Roy. Soc. W. Aust.* **20**: 103.
- COOKSON, I. C. (1946).—*Proc. Linn. Soc. N.S.W.* **71**: 49.
- COOKSON, I. C. (1947*a*).—*Proc. Linn. Soc. N.S.W.* **72**: 207.
- COOKSON, I. C. (1947*b*).—*Rep. Brit., Aust., N.Z. Antarctic Res. Exped. A* **2**: 127.
- COOKSON, I. C. (1950).—*Aust. J. Sci. Res. B* **3**: 166.
- CRANWELL, L. (1941).—*N.Z. J. Sci. Tech.* **22**: 1B.
- DALLIMORE, W., and JACKSON, A. B. (1948).—"Handbook of Coniferae." (Edward Arnold & Co.: London.)
- DEANE, H. (1925).—*Rec. Geol. Surv. Vict.* **4**: 492.
- ERDTMAN, G. (1948).—*Geol. Fören Stockh. Förh.* **70**: 265.
- VON ETTINGSHAUSEN, C. (1888).—*Mem. Geol. Surv. N.S.W. Palaeontol.* No. 2. 189 pp.
- FLORIN, R. (1931).—*K. Svenska VetenskAkad. Handl.* (3) **10**: 1-588.
- FLORIN, R. (1940).—*K. Svenska VetenskAkad. Handl.* (3) **19** (2): 1-107.
- FLORIN, R. (1951).—*Acta Hort. Berg.* **15** (11): 285.
- FRANCIS, W. D. (1929).—"Australian Rain-Forest Trees." 347 pp. (Government Printer: Brisbane.)
- JOHNSTON, R. M. (1888).—"A Systematic Account of the Geology of Tasmania." (Hobart.)
- KENDALL, M. W. (1949).—*Ann. Bot.* **13**: 151.
- KRAUSEL, R. (1949).—*Palaeontographica B* **89**: 83.
- MCAURTHUR, W. M. (1949).—Res. Rep. Geog. Lab. Univ. W. Aust. No. 5.
- VON MUELLER, F. (1883).—"Observations on New Vegetable Fossils of the Auriferous Drifts," 2nd Decade, Melbourne. (Geol. Surv. Vict.)
- SELLING, O. H. (1950).—*Svensk Bot. Tidskr.* **44**: 551.
- SEWARD, A. C., and CONWAY, V. (1934).—*Ann. Bot.* **48**: 715.
- SEWARD, A. C., and FORD, S. O. (1906).—*Philos. Trans. B* **198**: 365.
- SINGLETON, F. A. (1941).—*Proc. Roy. Soc. Vict.* **53** (1): 1.
- SWAIN, E. H. F. (1928).—"The Timbers and Forest Products of Queensland." 500 pp. (Government Printer: Brisbane.)
- THIBOUT, E. (1896).—"Recherches sur l'appareil mâle des Gymnospermes." (Lille.)
- THOMAS, D. E., and BARAGWANATH, W. (1949).—*Min. Geol. J. Melb.* **3**: 28.
- THOMSON, R. B. (1905).—*Science* **22**: 88.
- WODEHOUSE, R. (1935).—"Pollen Grains." (New York.)

EXPLANATION OF PLATES 1-6

The photographs are the work of I. Cookson and all figures are from untouched negatives. The letters N.M.V. before a specimen number refer to the collection of the National Museum of Victoria. Duplicate specimens, where available, will be deposited in the Geological Department, British Museum (Natural History).

PLATE 1

Araucaria lignitici n.sp.

Fig. 1.—Portion of a shoot. Natural size. (N.M.V. P.15254.)

Fig. 2.—Portions of two shoots. Natural size. (N.M.V. P. 15265.)

Fig. 3.—Small fragment of a shoot with shorter, broader leaves. x2 (N.M.V. P. 15256.)

Fig. 4.—Portion of a shoot. $\times 1\frac{1}{2}$. (N.M.V. P. 15255.)

Fig. 5.—Entire cuticle of a leaf opened out to show the distribution of stomata on the upper and lower surfaces. $\times 17$.

Fig. 6.—A stoma with its associated subsidiary and encircling cells, in transverse section. $\times 400$.

Fig. 7.—Cuticle of upper surface of a leaf. $\times 200$.

PLATE 2

Fig. 8.—*Araucaria lignitici*. Portion of a male cone. Natural size. (N.M.V. P. 18266.)

Fig. 9.—*A. lignitici*. The same cone. $\times 3$.

Fig. 10.—*A. lignitici*. Portions of another male cone. $\times 2$. (N.M.V. P. 15258.)

Fig. 11.—*A. lignitici*. Portions of a male cone. $\times 2\frac{1}{2}$. (N.M.V. P. 15259.)

Fig. 12.—*A. lignitici*. Cuticle of a bract opened out. $\times 15$.

Fig. 13.—*A. lignitici*. Lower cuticle of the lamina of a male sporophyll. $\times 40$.

Fig. 14.—*A. lignitici*. Pollen grain from a male cone. $\times 500$.

Fig. 15.—*A. lignitici*. Another pollen grain from a male cone. $\times 500$.

Fig. 16.—*Araucaria columnaris*. An oblique section through the exine of an acetolysed pollen grain. $\times 1220$.

Fig. 17.—*A. columnaris*. Surface view of exine at a high focus. $\times 1200$.

Fig. 18.—*A. columnaris*. The same area at a low focus. $\times 1200$.

PLATE 3

Fig. 19.—*Araucaria lignitici*. A female cone-scale. Natural size. (N.M.V. P. 15261.)

Fig. 20.—*A. lignitici*. Another cone-scale. Natural size. (N.M.V. P. 15262.)

Fig. 21.—*A. lignitici*. A third example showing full length of the spinous process at the distal end of the scale. $\times 3$. (N.M.V. P. 15263.)

Fig. 22.—*A. lignitici*. Upper cuticle of distal region of cone-scale and ligule. $\times 12$.

Fig. 23.—Upper cuticle from above the seed. $\times 70$.

Fig. 24.—Cuticle of the lateral wing. $\times 70$.

Fig. 25.—*Araucaria beccarii*. Cuticle of the upper surface above the seed, showing long, narrow epidermal cells with thin, unpitted walls. $\times 70$.

Fig. 26.—*A. cunninghamii*. Cuticle of the upper surface above the seed, showing prominent groups of "sister cells." $\times 70$.

Fig. 27.—*A. columnaris*. Cuticle of the upper surface above the seed, showing unevenly thickened, strongly pitted cell walls. $\times 140$.

PLATE 4

Agathis yallournensis n.sp.

Fig. 28.—An almost complete leaf. Natural size. (N.M.V. P. 15266.)

Fig. 29.—Portion of another leaf. A small globule of resin is seen in transverse section on the right-hand margin of the leaf. Natural size. (N.M.V. P. 15267.)

Fig. 30.—Portion of a wide leaf. Natural size. (N.M.V. P. 15268.)

Fig. 31.—Another leaf showing an obtuse apex. Natural size. (N.M.V. P. 15269.)

Fig. 32.—A small complete leaf. Natural size. (N.M.V. P. 15270.)

Fig. 33.—Cuticle of upper epidermis. $\times 100$.

Fig. 34.—Cuticle of lower epidermis. $\times 100$.

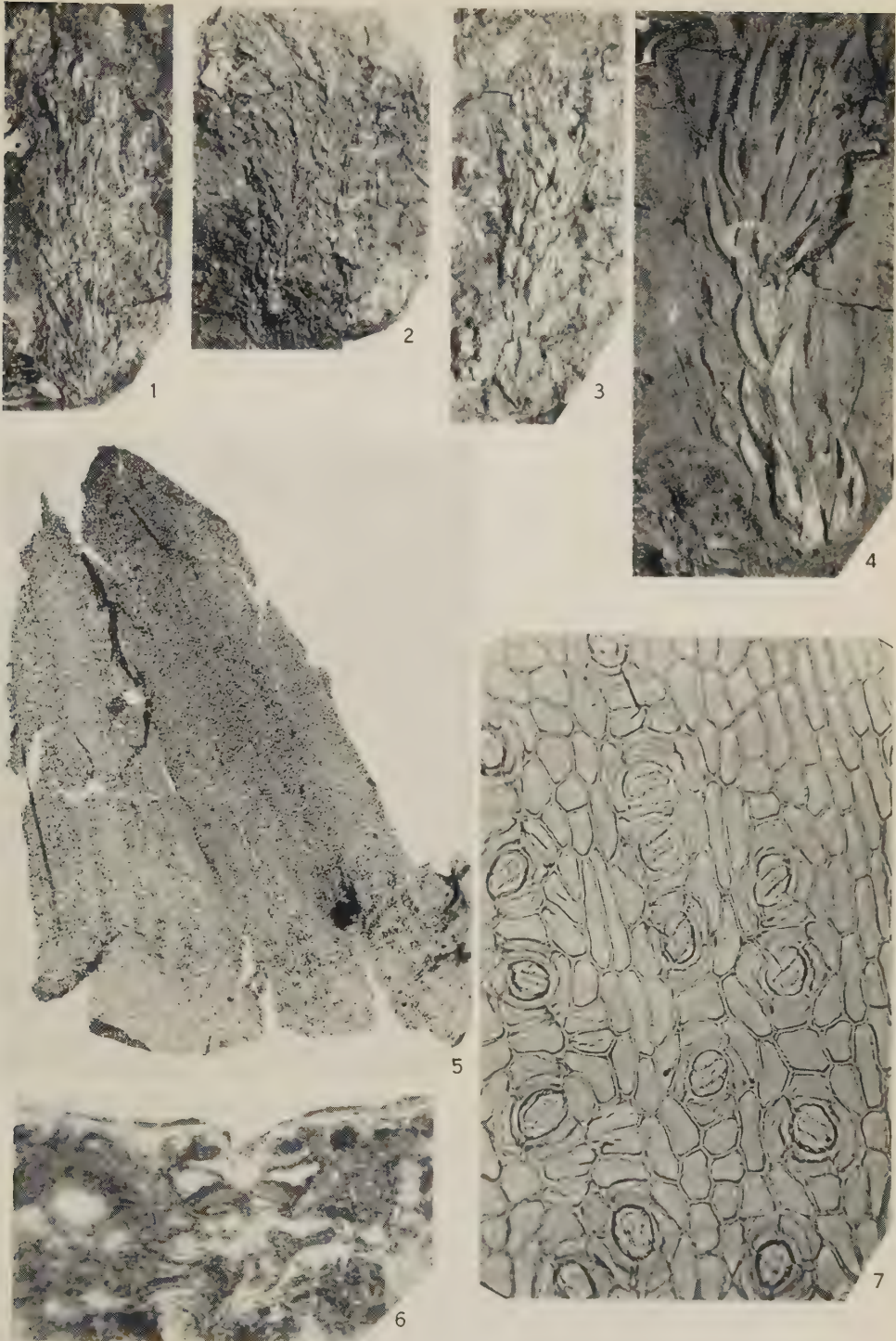
Fig. 35.—Cells of upper epidermis showing pitted cell walls. $\times 400$.

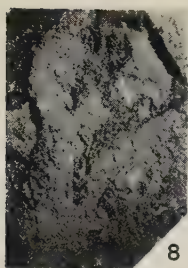
Fig. 36.—Lower epidermis showing two stomatal groups. $\times 400$.

PLATE 5

Fig. 37.—*Agathis yallournensis*. Transverse section of a leaf. $\times 120$.

Fig. 38.—*A. yallournensis*. Transverse section of a leaf showing a sclereid. $\times 400$.

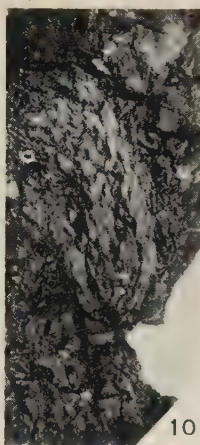




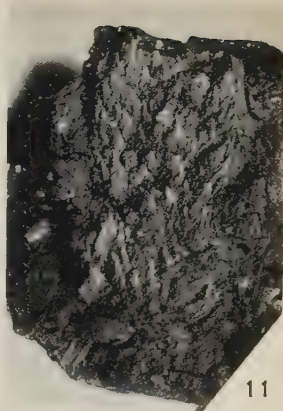
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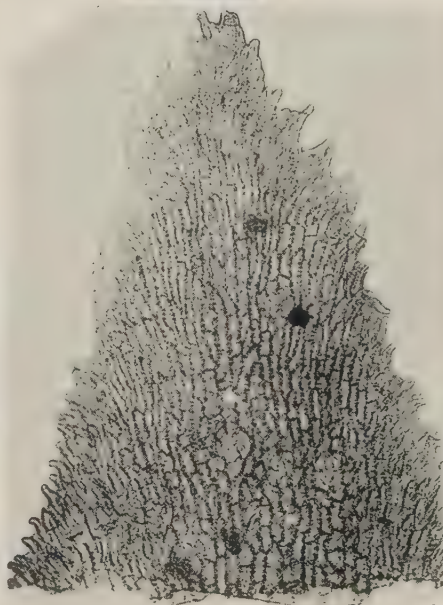
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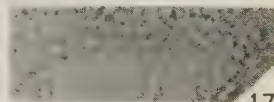
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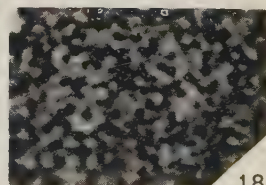
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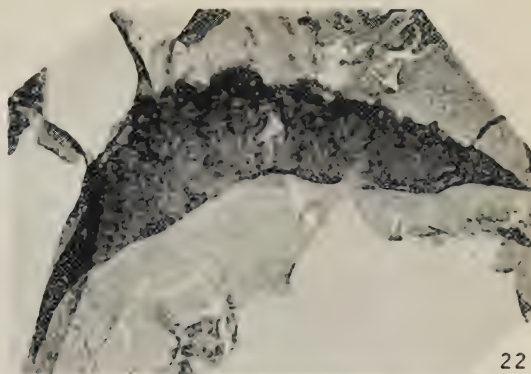
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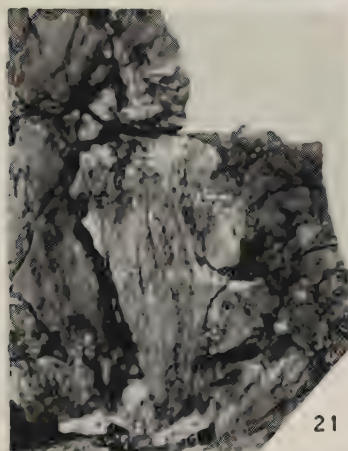
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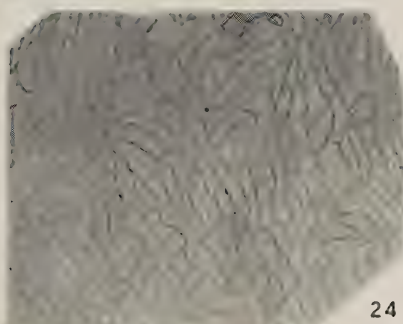
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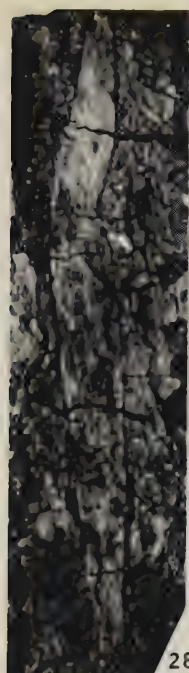
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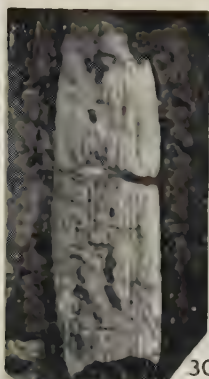
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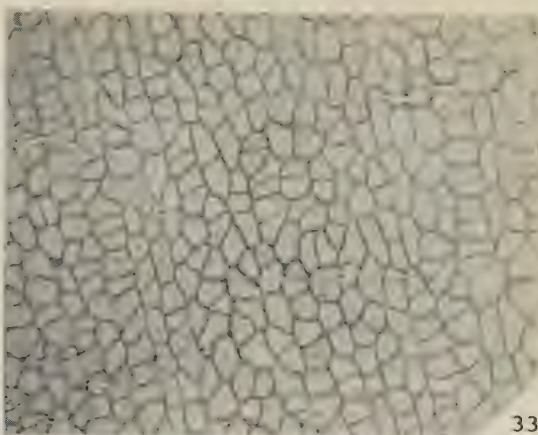
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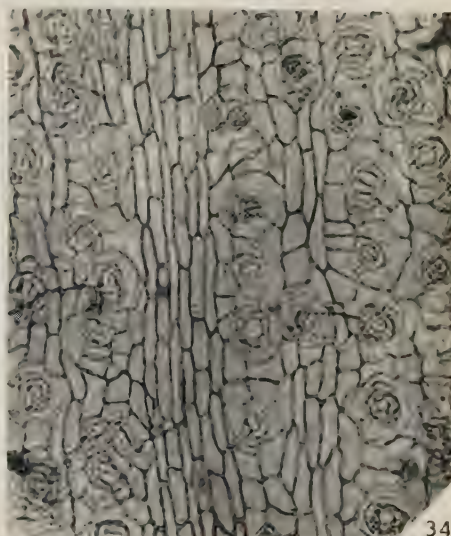
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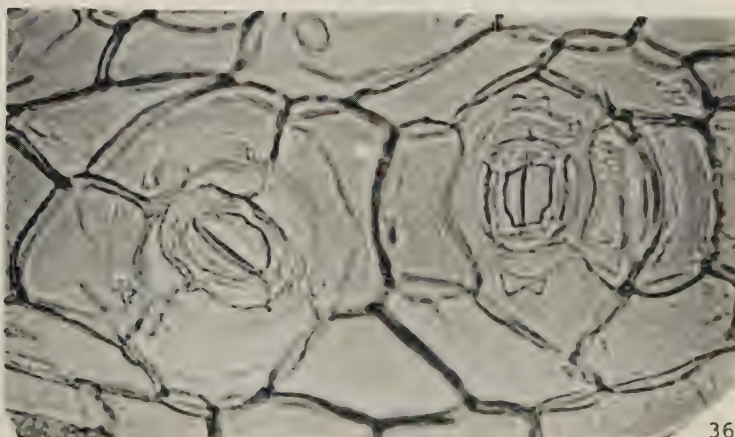
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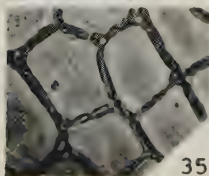
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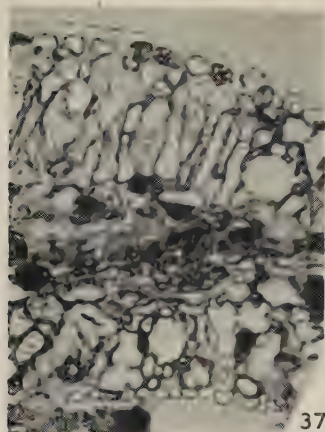
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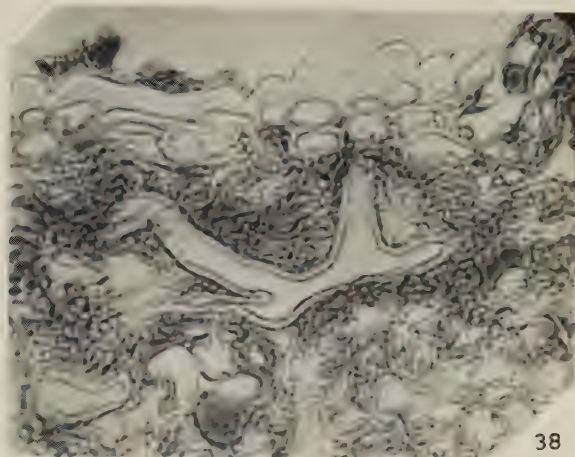
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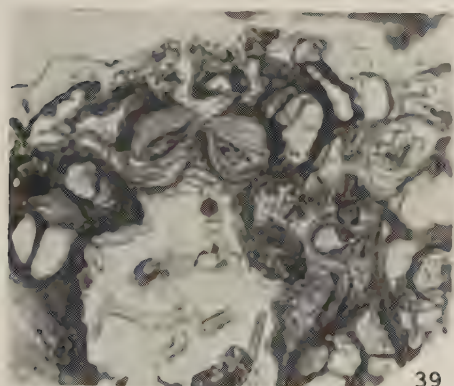
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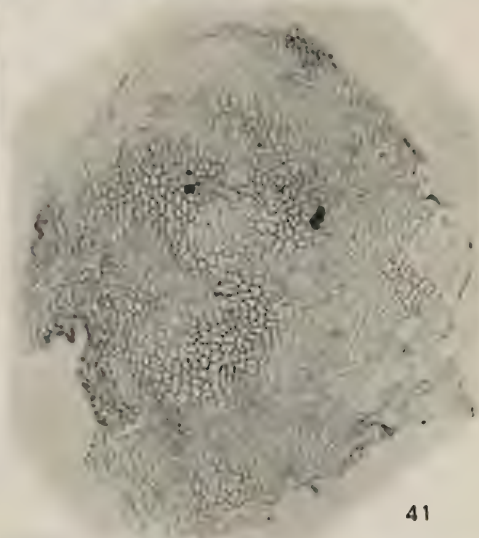
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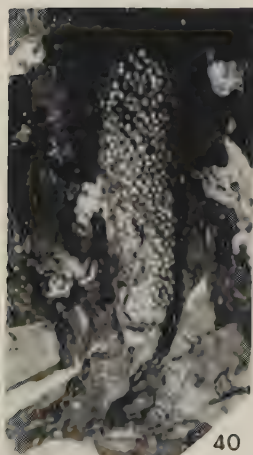
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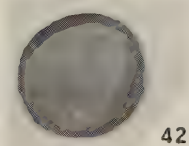
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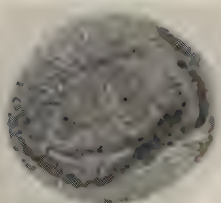
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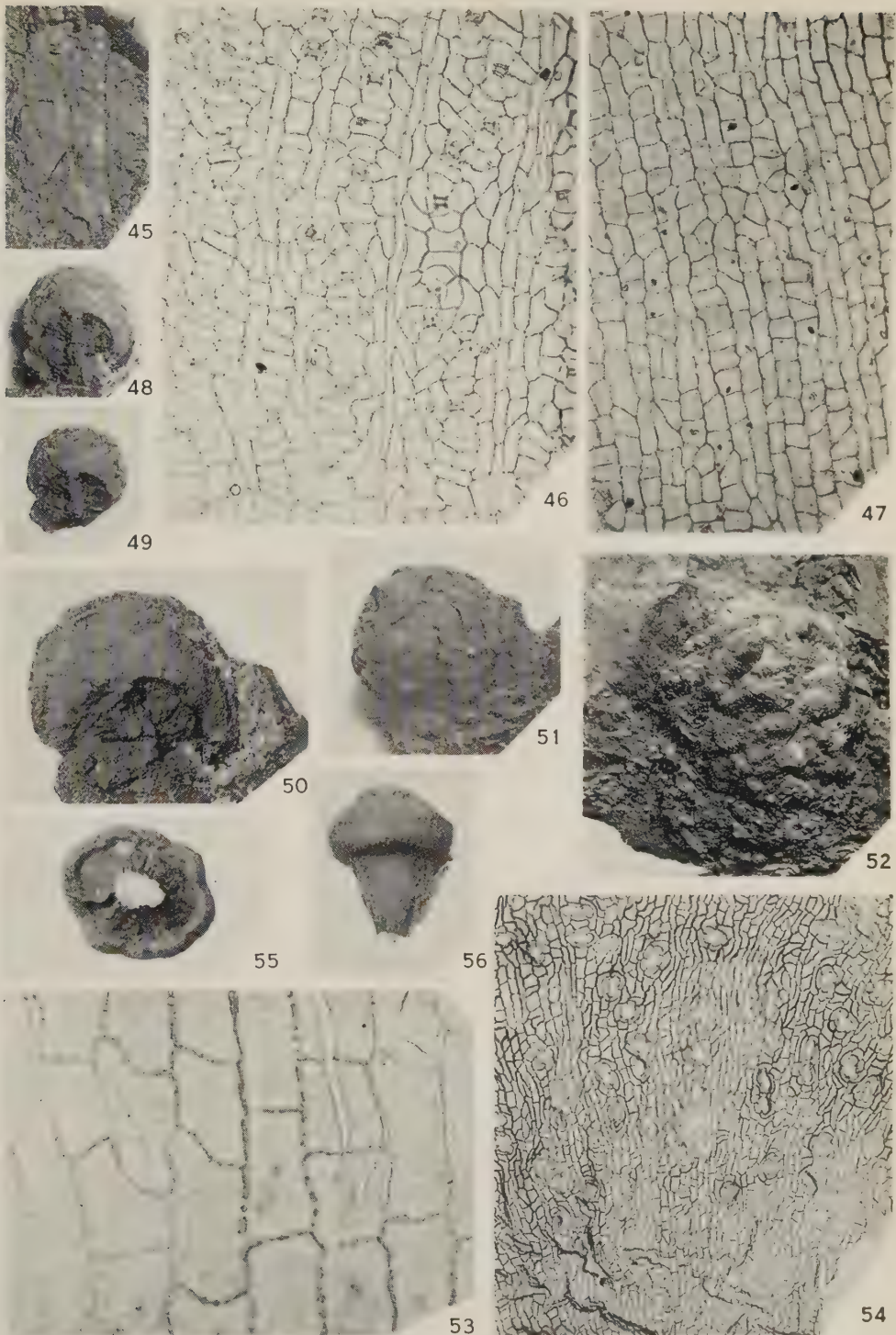


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AUSTRALIAN TERTIARY ARAUCARIACEAE



- Fig. 39.—*A. yellournensis*. Transverse section of a leaf showing a stoma, the thick-walled guard-cells of which are overarched by subsidiary and encircling cells. x400.
 Fig. 40.—*A. yellournensis*. A male cone. Natural size. (N.M.V. P. 15271.)
 Fig. 41.—*A. yellournensis*. The lamina of a male sporophyll. x45.
 Fig. 42.—*A. yellournensis*. A small pollen grain from the male cone shown in Figure 40. x500.
 Fig. 43.—*A. yellournensis*. An average-sized pollen grain from the same cone. x500.
 Fig. 44.—*Agathis* cf. *intermedia* Ett. Specimen from Cape Riche, Western Australia, identified as such by Chapman and Crespin. x2.

PLATE 6

- Fig. 45.—*Agathis parwanensis*. A leaf fragment. Natural size. (N.M.V. P. 15272.)
 Fig. 46.—*A. parwanensis*. Cuticle of lower epidermis. x100.
 Fig. 47.—*A. parwanensis*. Cuticle of upper epidermis. x100.
 Fig. 48.—*Agathis yellournensis*. Immature female cone from below showing the insertion of cone-scales on the axis of the cone. Natural size. (N.M.V. P. 15273.)
 Fig. 49.—*A. yellournensis*. The same specimen more fully exposed as the result of the removal of a small piece of coaly matrix from the right-hand side. Slightly less than natural size.
 Fig. 50.—*A. yellournensis*. The same view as in Figure 48. x2.
 Fig. 51.—*A. yellournensis*. The reverse surface of the same specimen. x2.
 Fig. 52.—*A. yellournensis*. Another female cone. x1½.
 Fig. 53.—*A. yellournensis*. Upper cuticle of the distal region of a cone-scale. x280.
 Fig. 54.—*A. yellournensis*. Lower cuticle of the same region. x70.
 Fig. 55.—*A. australis*. Upper portion of young female cone, seen from below.
 Fig. 56.—*A. australis*. Under surface of immature female cone-scale.

APPENDIX I

SOURCE OF MATERIAL OF LIVING SPECIES

ARAUCARIA	LEAVES
<i>A. balansae</i>	Colln. J. T. Buchholz, No. 1355. Plaine des Lacs, New Caledonia.
<i>A. beccarii</i>	Herb. Mus. Brit. No. 5749. L. S. Gibbs. Angi Lakes, Dutch NW. New Guinea.
<i>A. bernieri</i>	Colln. J. T. Buchholz, No. 1562. Plaine des Lacs, New Caledonia.
<i>A. biramulata</i>	Colln. J. T. Buchholz, No. 1691. Foret de Mai, Plaine des Lacs, New Caledonia.
<i>A. columnaris</i>	Colln. J. T. Buchholz, No. 1666. Isle of Pines. Nat. Herb. Victoria. New Caledonia. Melbourne Bot. Gard.
<i>A. cunninghamii</i>	Nat. Herb. Victoria. Queensland. Melbourne Bot. Gard. System garden, Bot. Dept., Univ. of Melbourne.
<i>A. excelsa</i>	Colln. J. T. Buchholz, No. 1599. Norfolk I. Melbourne Bot. Gard.
<i>A. humboldtensis</i>	Colln. J. T. Buchholz, No. 1686. Mt. Mou, New Caledonia.
<i>A. intermedia</i>	Kew Herb., No. 1276. Vieillard, New Caledonia.
<i>A. montana</i>	Colln. J. T. Buchholz, No. 1603. Mt. Ouli, N. of Table Mt., New Caledonia.
<i>A. muelleri</i>	Colln. J. T. Buchholz, No. 1207. New Caledonia.

APPENDIX I (*Continued*)

- A. rulei* Colln. J. T. Buchholz, No. 1457. Mt. Mou, New Caledonia.
Melbourne Bot. Gard.
Kew Herb. Balansa. Kanala, New Caledonia.

MALE CONES

- A. balansae* Queensland Herb., No. 2121. C. T. White. det. ver. A. Arb. Baie des Pirogues, New Caledonia.
Service des Eaux et Forêts, New Caledonia.
- A. beccarii* Queensland Herb., No. 11175. L. J. Brass and C. Versteigh. Balim River, Dutch New Guinea.
- A. bernieri* Colln. J. T. Buchholz, No. 1562. Plaine des Lacs, New Caledonia.
- A. biramulata* Colln. J. T. Buchholz. Foret du Mois de Mai, Plaine des Lacs, New Caledonia.
- A. columnaris* Queensland Herb., No. 2290. C. T. White. Noumea (cult.).
Herb. Brit. Mus., No. 1278. Vieillard. "Ad Tante Novae Caledoniae."
Colln. J. T. Buchholz. Southern New Caledonia.
Service des Eaux et Forêts, New Caledonia (pollen only).
- A. cunninghamii* Queensland Herb., No. 6647. C. E. Hubbard and C. W. Winders. Magnetic I.
- A. excelsa* Queensland Herb., No. 11091. C. T. White. Cleveland, Brisbane.
- A. montana* Colln. J. T. Buchholz, No. 1603. Mt. Ouli, N. of Table Mt., New Caledonia.
- A. muelleri* Kew Herb., No. 188. M. Balansa. New Caledonia.
- A. rulei* Kew Herb., No. 2167. J. G. Veitch. New Caledonia.
Colln. J. T. Buchholz. Mt. Mou, New Caledonia.

FEMALE CONE-SCALES

- A. balansae* Colln. J. T. Buchholz, No. 1608. New Caledonia.
- A. beccarii* Herb. Brit. Mus., No. 5749. L. S. Gibbs. Angi Lakes, Dutch NW. New Guinea.
- A. bernieri* Colln. J. T. Buchholz. Plaine des Lacs, near Pirogues R., New Caledonia.
- A. biramulata* Colln. J. T. Buchholz, No. 1691. Foret de Mai, Plaine des Lacs, New Caledonia.
- A. columnaris* Colln. J. T. Buchholz, No. 1666. Isle of Pines.
Nat. Herb. Victoria. New Caledonia.
Museum, Bot. Dept., Univ. of Melbourne.
- A. cunninghamii* System garden, Bot. Dept., Univ. of Melbourne.
- A. excelsa* Museum, Bot. Dept., Univ. of Melbourne.
- A. humboldtensis* Colln. J. T. Buchholz, No. 1206. Pic du Rocher, New Caledonia.
- A. muelleri* Colln. J. T. Buchholz, No. 1207, New Caledonia.
- A. rulei* Colln. J. T. Buchholz, No. 1457. Mt. Mou, New Caledonia.

AGATHIS

- A. alba*
- A. australis*

LEAVES

- Nat. Herb. Victoria. Java.
Colln. R. Holttum. Penang Hill.
- Herb. Auckland Inst. and Mus., N.Z. L. M. Cranwell. Henderson, near Auckland, N.Z.

APPENDIX I (*Continued*)

- A. brownii* ' Nat. Herb. Victoria. Wide Bay, N. of Gympie, Queensland (type area).
Melbourne Bot. Gard.
Sydney Bot. Gard.
Brisbane Bot. Gard.
- A. celebica* Neth. Ind. For. Serv., No. bb 31503. Manado, Posa, Bantjea.
- A. flavescens* Colln. R. Holttum. Gunong Tahan.
- A. lanceolata* Service des Eaux et Forêts, New Caledonia.
Nat. Herb. Victoria.
- A. microstachya* Queensland Herbarium, Atherton Tableland.
- A. moorei* Nat. Herb. Victoria.
Sydney Bot. Gard.
- A. obtusa* Nat. Herb. Victoria.
- A. ovata* Nat. Herb. Victoria.
Colln. J. T. Buchholz, No. 1700. Plaine des Lacs, New Caledonia.
- A. palmerstoni* Nat. Herb. Victoria. Mt. Bartlefrère, N. Queensland (type specimen and material from type area).
Brisbane Herb., Mt. Molloy, N. Queensland; Parish of Barron, N. Queensland; Ravenshoe, N. Queensland; Atherton District, N. Queensland.
- A. philippinensis* Neth. Ind. For. Serv., No. bb 28287. Boven Burveang, Pandok Bakarve.
- A. regia* Neth. Ind. For. Serv., No. bb 24584. Molukken, Pilowe, Eil Morotai.
- A. vitiensis* Nat. Herb. Victoria.

MALE CONES

- A. alba* Colln. R. Holttum. Penang Hill.
- A. australis* Herb. Auckland Inst. and Mus., N.Z. L. M. Cranwell. Henderson, near Auckland, N.Z.
- A. brownii* Brisbane Bot. Gard.
- A. lanceolata* Queensland Herb., No. 2064. C. T. White. Mt. Mou, New Caledonia.
Arnold Arboretum. Baie des Pirogues, New Caledonia.
- A. microstachya* Queensland Herb., Atherton Tableland.
- A. moorei* Sydney Bot. Gard.
- A. obtusa* Queensland Herbarium, No. 282. S. F. Kajewski. Nerndu, Dillon's Bay, Erromanga, New Hebrides.
- A. ovata* Queensland Herb., No. 2284. C. T. White. Mountains near Dumbea, New Caledonia.
- A. palmerstoni* Queensland Herb., No. 2421. L. J. Brass. Mt. Molloy, N. Queensland.
- A. philippinensis* Kew Herb., No. 805. T. E. Borden. Lamas R., Mt. Meriveles, Bataan, Luzon.
- A. vitiensis* Kew Herb., No. 15273. D. Degener. Viti Leva.

FEMALE CONE-SCALES

- A. australis* Herb. Auckland Inst. and Mus., N.Z.
- A. brownii* Melbourne Bot. Gard.
- A. lanceolata* Colln. J. T. Buchholz, No. 1604. Near Canala, New Caledonia.
- A. moorei* Sydney Bot. Gard.
- A. ovata* Colln. J. T. Buchholz, No. 1700. Plaine des Lacs, New Caledonia.
- A. palmerstoni* Nat. Herb. Victoria. Mt. Bartlefrère, N. Queensland (type locality).
- A. philippinensis* Dist. A. E. Elmer. Philippine I.

AN AGE-INDUCED VARIATION IN SUSCEPTIBILITY TO VIRUS X IN *NICOTIANA TABACUM* L.

By E. M. HUTTON*

[Manuscript received April 20, 1951]

Summary

Stage of maturity influences the susceptibility of *N. tabacum* to virus X. This phenotypic variation is evidenced by a discontinuity of X infection in the inoculated leaves and a lack of systemic invasion of plants inoculated at a late growth stage compared with a complete infection of the inoculated leaves and a rapid systemic invasion of plants inoculated at an early growth stage. Aging, even under conditions of very low nutrition, of plants inoculated at an early growth stage had no influence on their virus status. There was no indication that a virus X inhibitor was responsible for the reduced susceptibility to infection of old tobacco plants.

In view of the phenotypic variation induced by increasing maturity it is necessary to take into account this easily controllable factor when evaluating progenies in programmes covering the genetics of virus resistance.

I. INTRODUCTION

During the course of a genetical study of virus X resistance in the seedling progenies of a number of potato (*Solanum tuberosum* L.) crosses, the standard Brownell type X (Bald and White 1942) used for inoculation was cultured in either tobacco (*Nicotiana tabacum* L.) or *Datura stramonium* L. Although *D. stramonium* cultures were invariably satisfactory the results of the inoculum from the tobacco cultures were sometimes aberrant. This was evidenced by the failure of some of the control plants of the virus-free variety, Factor, to become infected. Normally young plants of this variety are completely susceptible to hand inoculation with virus X. It was concluded that virus X was either absent or in very low concentration in the tobacco plants responsible for the aberrant results.

Investigation of the problem revealed an interesting undescribed relationship between maturity of tobacco plants at time of inoculation with virus X and extent of systemic development of this virus. It is considered that the experiments on this relationship described in the present paper assist in the understanding of the phenotypic reactions that result in the resistance of plants to viruses. The phenotypic reaction of a plant to a virus is determined by the interaction between the genotype and the environment and one of the most important components of the environment in this connection is temperature. The effect of temperature on the reaction to the mosaic virus of tobacco hybrids developed

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from known genotypes has been described by McKinney and Clayton (1945). That non-environmental factors like plant vigour and maturity also influence the reaction to viruses of phenotypes derived from virus Y-resistant genotypes has been shown by Hutton (1948). Bawden and Roberts (1947), in their experiments on the influence of light on the susceptibility of tobacco, *Nicotiana glutinosa*, and tomato to several of a group of four viruses, noticed a gradient of increasing susceptibility from the oldest to the youngest leaves on the same plants and considered that in older plants and leaves this could be due to the accumulation of photosynthetic products inhibitory to virus multiplication. They did not work with virus X and used relatively young plants for all their experiments. Such relationships as have been described emphasize the need for a biochemical understanding of phenotypic reactions to viruses as a basis for the genetical study of virus resistance in plants and for the development of new genetical methods for solving these problems.

II. MATERIALS AND METHODS

For all the experiments described virus X was cultured in *D. stramonium*. This plant was used also as the main indicator for the presence of virus X. In the final experiments with Turkish tobacco and in the tests for the presence of an inactivating substance in old tobacco plants where a quantitative measure of virus was required, the globe amaranth (*Gomphrena globosa* L.) as described by Wilkinson and Blodgett (1948) was used.

In the first two series of experiments Brownell type X was used but in the later ones a virulent strain of X was employed to obviate possible complications from avirulent strains normally present in the Brownell type. Inoculum was prepared by macerating the leaf tissue in a mortar with a pestle at the constant proportion of 1 : 20 by weight with water. Where the pattern of virus development was being followed in the inoculated leaves 1 : 40 inoculum was used. All inoculations were done with a ground-glass spatula after dusting the leaves with fine carborundum powder.

III. AGE OF TOBACCO PLANTS AT INOCULATION AND DEGREE OF SYSTEMIC INVASION BY POTATO VIRUS X, USING TOBACCO VARIETY KENTUCKY 41A

Preliminary experiments with the tobacco variety Kentucky 41A indicated clearly that young plants invariably became systemically infected after inoculation of tip leaves with virus X. On the other hand, plants at or close to the flowering stage rarely became invaded systemically when inoculated. Even the introduction of virus by grafting did not result in a full systemic invasion of old plants. This was demonstrated by an experiment in which two young tobacco plants four weeks from transplanting, and six old tobacco plants 14 weeks from transplanting, were grafted towards the base with Brownell potato scions. At monthly intervals over a period of four months portions of the tip leaf and of the basal leaf of the tobacco plants were inoculated separately to duplicate plants of *D. stramonium*. The young plants became systemically infected with virus

X within the first month. In the old plants there was no movement of Brownell X to the tips but a gradual invasion of all the basal leaves within three months.

A detailed experiment extending over 10 months was done to discover more about the principles involved in the apparent resistance of old tobacco plants to systemic invasion by virus X. Fifteen young tobacco plants were transplanted and divided equally into five groups. At 4, 8, 14, 19, and 26 weeks respectively after transplanting, one of these groups was inoculated once on the young tip leaves with Brownell X. When inoculated the approximate heights of the five groups were 10, 24, 35, 41, and 45 in. respectively, the third group being near flowering, and the last two groups at the flowering stage. At 10-weekly intervals after plants were inoculated, portions of the youngest tip leaf and of the basal leaf were each inoculated separately to duplicate plants of *D. stramonium*. Where the basal leaf had yellowed the next lowest green leaf was used. If the indicator plants remained free of symptoms they were reinoculated with a virulent strain of X to test for the presence of masked strains.

TABLE 1
EFFECT OF AGE OF TOBACCO PLANT AT INOCULATION ON SYSTEMIC INVASION BY
VIRUS X AS SHOWN BY TRANSFERS TO *DATURA STRAMONIUM* L.

Group	Tobacco Plant Nos.	No. of Weeks After Transplanting when Inoculated	Results of Transfers to <i>D. stramonium</i> * at 10-weekly Intervals after Inoculation of Tobacco Plants							
			1		2		3		4	
			Tip	Base	Tip	Base	Tip	Stem	Tip	Stem
1	1,2,3	4	+	+	+	+	+		+	+
2	4,5,6	8	4 + 5&6 O	+	4 + 5&6 O	+	+	+	+	+
3	7,8,9	14	O	O	O	O	O	O		
4	10,11,12	19	O	O	O	O	Stem O			
5	13,14,15	26	O	O	O	O	Stem O			

* + Signifies presence of, and O absence of, virus X in *D. stramonium*.

In no case was a masked strain found. After the fourth transfer from their tip leaves to *D. stramonium*, the first group of tobacco plants inoculated were cut back to a height of 14 in. above the soil in the pot, a small piece of stem at this height then being inoculated to duplicate plants of the indicator. The same procedure was adopted for the second and third groups of tobacco plants inoculated after the third transfer to *D. stramonium*, and for the fourth and fifth groups after the second transfer. Before cutting back, the tobacco plants of the first and second groups were 24-30 in. in height while the rest of the groups varied from 48 to 60 in. above the pots. These differences were in themselves striking evidence of systemic invasion by virus X in the first two groups and its retarded development in the last three groups.

From the results in Table 1 it can be seen that systemic invasion by virus X occurred in the tobacco plants inoculated four weeks after transplanting, while one of those inoculated eight weeks after transplanting was readily invaded, and in the other two plants of this second group systemic development of the virus was retarded and did not occur until 20-30 weeks after inoculation. Evidently in these latter two plants physiological conditions were unfavourable to the development of virus X, and the fact that systemic invasion did not occur at all in the plants inoculated 14, 19, and 26 weeks after transplanting indicates that at eight weeks after transplanting the factors inhibiting systemic virus development had almost outbalanced those encouraging it. In Table 1 it is apparent that, once systemic invasion with virus X has occurred in young plants like those in group 1, their subsequent aging does not influence their virus status. In addition it is of interest to note that, following the inoculations from stem pieces obtained by cutting back the plants, the young leaf regrowth was inoculated to duplicate plants of *D. stramonium* with similar results to those obtained from the stem material.

IV. EFFECT OF NUTRITION ON THE VIRUS STATUS OF TOBACCO PLANTS INFECTED WITH X AT AN EARLY GROWTH STAGE

As the previous experiments had shown that tobacco plants in their later growth stages were resistant to systemic invasion by virus X it was of interest to see whether it was possible to influence the virus status of old plants that had been infected at an early growth stage. Table 1 indicated that aging in itself did not influence the virus content of plants inoculated early in life so it was decided to use a combination of aging and low level of nutrition.

Ten young tobacco plants of the variety Kentucky 41A were inoculated with Brownell type X, and six weeks later when they were strongly mottled they were transplanted to washed river sand and given water only. At monthly intervals over a 12-months period, portions of the tip leaves of each plant were inoculated to duplicate *D. stramonium* plants. In spite of the severe effect of aging at a very low level of nutrition on growth and metabolism the indicator plants always showed the presence of virus X. None of the tobacco plants grew more than 12 in. high and the leaves were dwarfed and intensely chlorotic. Under these conditions the symptoms of virus X disappeared but reappeared for a short period after an application of potassium nitrate half way through the experiment.

It is apparent that, when virus X development becomes an integral part of the protein metabolism of a young tobacco plant, aging in rich potting soil as in the experiment of Table 1, or aging under conditions of very low nutrition do not effect a change in the phenotype. However, if a plant is aged before inoculation with virus X, a phenotypic variation is induced resulting in a physiological state inhibitory to virus development.

V. PATTERN OF VIRUS X DEVELOPMENT IN OLD AND YOUNG PLANTS OF TOBACCO VARIETY KENTUCKY 41A

In order to gain some understanding of the age-induced phenotypic variation in virus X susceptibility in the tobacco variety Kentucky 41A, the pattern of virus development in the inoculated leaves of young and old plants was investigated. Three young, vigorously growing plants 9-10 in. high at four weeks after transplanting and two old plants 14 weeks from transplanting and at the flowering stage and 35-40 in. high were selected. Inoculum of a virulent strain of X was prepared by macerating 1 g. of leaf tissue in 40 ml. of water. Two leaves of each of the young plants and six leaves of equivalent size on each of the old plants were tagged and inoculated. Five days after inoculation a systemic mottle had developed in the young plants, but no systemic symptoms were present in the old plants. Twelve days after inoculation young tip leaves of each of the five tobacco plants were inoculated to duplicate *D. stramonium* plants.

TABLE 2
EXTENT OF DEVELOPMENT OF A VIRULENT STRAIN OF X IN LEAVES OF OLD AND YOUNG KENTUCKY 41A TOBACCO PLANTS AFTER THEIR INOCULATION, AS SHOWN BY A TISSUE SAMPLING METHOD

Tobacco Plants			Number of Tissue Discs Out of 12 Giving Virus X	
No.	Weeks from Trans-planting	Inoculated Leaves Used	12 Days after Inoculation	30 Days after Inoculation
1	14	Top	6	11
		Lower	8	10
2	14	Top	9	11
		Lower	5	11
3	4	One	12	12
4	4	One	12	12
5	4	One	12	12

At the same time as the tip leaves were sampled, 12 discs of tissue 4 mm. in diameter were removed with a cork borer from each of seven inoculated leaves, four of these being on the two old plants and the rest of the leaves being distributed among the three young plants. The tobacco leaves were of similar size and ranged in area from 225 to 280 sq. cm. Plate 1 shows the 12 discs removed from one of the tobacco leaves and the manner in which they were distributed. Each disc of tissue was inoculated to a single *D. stramonium* plant.

A month after inoculation of the two old and three young tobacco plants a similar series of discs was removed from another set of their inoculated leaves. As before, portions of the young tip leaves were inoculated to duplicate *D. stramonium* plants at the same time as the discs of tissue were inoculated to single plants of this indicator. The results of the two series of inoculations are given in Table 2.

It will be seen in Table 2 that, 12 days after inoculation, none of the discs taken from the inoculated leaves of the young plants was free of X whereas 25-58 per cent. of those taken from the inoculated leaves of the old plants were free of X as measured by infection in *D. stramonium*. At this stage a definite systemic mottle was present in the young plants while no systemic movement of X was detected in the old plants. Thirty days after inoculation a similar position applied in the young plants but in the old plants a further development of virus X had taken place so that only 8-16 per cent. of the tissue discs from the inoculated leaves were free of X. Even this indicates considerable discontinuity of virus in the leaves of the old plants and fits into the general picture of an age-induced phenotypic variation in susceptibility to virus X. As before, no systemic movement of virus X to the young tip leaves had occurred in the old plants.

After these results were obtained, the stems of all the tobacco plants were cut into six equal lengths, each of which was inoculated to duplicate *D. stramonium* plants. The position with respect to presence or absence of X in these stem portions is shown in Table 3.

TABLE 3
VIRUS X STATUS OF THE STEMS OF OLD AND YOUNG KENTUCKY 41A TOBACCO PLANTS AT SUCCESSIVE SITES ALONG THEIR LENGTH FOLLOWING LEAF INOCULATION

Tobacco Plants							
No.	Weeks from Trans- planting	Base		Mid Stem		Tip	
		1	2	3	4	5	6
1	14	O	+	+	+	O	O
2	14	O	O	O	O	O	O
3, 4, 5	4	+	+	+	+	+	+

+ Signifies presence of, and O absence of, virus X in *D. stramonium*.

From the evidence in Table 3 it is apparent that inhibition of virus X multiplication occurred in the stems of the two old tobacco plants inoculated on the leaves with virus X 14 weeks after transplanting. In plant 1 the virus had moved from the inoculated leaves into the corresponding stem areas, resulting in a limited development in these areas without migration to the rest of the stem. In plant 2 movement of virus X from the inoculated leaves may not have occurred, and if it did, there was a total inhibition of its development in the stem. As would be expected from the previous results, a free development of virus X occurred in the stems of the young plants 3, 4, and 5. It is thus clear from the results of Tables 2 and 3 that inhibition of virus X multiplication is common to both stem and leaf tissue of tobacco plants inoculated 14 weeks from transplanting.

VI. VIRUS X DEVELOPMENT IN A TURKISH VARIETY OF TOBACCO

As all the previous experiments were done with the tobacco variety Kentucky 41A it was necessary to compare the results of virus X inoculations in another variety with an inherently different phenotype. The Turkish type of tobacco, with its large number of short leaves and compact growth habit, was chosen as being inherently different from Kentucky 41A, with its fewer larger leaves and spreading growth.

Six flowering Turkish tobacco plants averaging 56 in. high, which had been transplanted 14 weeks earlier were each inoculated on six leaves with virulent X. With plants 1, 2, and 3 the inoculated leaves were along the lower half of the stem and in plants 4, 5, and 6 they were along the upper half of the stem. Three young Turkish tobacco plants four weeks from transplanting, and averaging 12 in. high, were each inoculated on three leaves as controls. A month later three or four discs of tissue 4 mm. in diameter were removed from a proportion of the inoculated leaves with a cork borer. All the inoculated leaves could not be sampled because the reaction to virus X of a number of them had resulted in the death and yellowing of a considerable area of the tissue. Only leaves of a normal green colour were sampled. Each disc of leaf tissue was macerated in two drops of water and inoculated to two small *D. stramonium* plants and half a globe amaranth leaf. After this sampling was completed, portions of the lowest uninoculated leaf and of the tip leaf were each inoculated to duplicate *D. stramonium* plants. The results are given in Table 4.

TABLE 4
RESULTS FROM THE VIRUS X INOCULATIONS OF OLD (NOS. 1-6) AND YOUNG (NOS. 7-9)
TURKISH TOBACCO PLANTS AS SHOWN BY TISSUE SAMPLING AND TRANSFER TO
D. STRAMONIUM AND GLOBE AMARANTH

Plant No.	No. of Leaves Sampled	No. of Tissue Discs Taken	No. of Tissue Discs Without X Indicated by <i>D. stramonium</i>	Mean No. Lesions per Half Leaf of Globe Amaranth	Systemic Movement of X (indicated by <i>D. stramonium</i>) to:	
					Basal Leaf	Tip Leaf
1	6	24	0	204	0	0
2	3	12	0	172	0	0
3	2	6	0	112	+	0
4	6	18	0	118	0	0
5	5	15	4	87	0	0
6	6	18	1	123	0	0
7,8,9	3	12	0	156	+	+

The results in Table 4 with Turkish tobacco agree with those described earlier in this paper for Kentucky 41A. It is apparent that systemic movement of virus X from the inoculated leaves of the old plants was rare and that virus development was inhibited in the leaves of plants 5 and 6 as evidenced by the

absence of virus X in 27 per cent. and 6 per cent. of the tissue discs respectively. The mean numbers of lesions per half leaf of globe amaranth have little significance but there may be a correlation with plant 5, which gave the greatest number of leaf discs free of X and the lowest mean number of globe amaranth lesions.

After the results of Table 4 were obtained, the old Turkish tobacco plants which then averaged 65 in. high and the young plants 24 in. high were stripped of their leaves and the stems cut into six equal pieces, each of which was inoculated to duplicate *D. stramonium* plants. The results are given in Table 5.

TABLE 5
VIRUS X STATUS OF THE STEMS OF OLD AND YOUNG TURKISH TOBACCO PLANTS
AT SUCCESSIVE SITES ALONG THEIR LENGTH FOLLOWING LEAF INOCULATION

Plant No.	Base		Mid Stem		Tip	
	1	2	3	4	5	6
1	O	+	O	O	O	O
2	O	O	O	O	O	O
3	O	+	O	O	O	O
4	O	O	O	+	+	O
5	O	O	O	O	O	O
6	O	O	O	+	+	+
7,8,9	+	+	+	+	+	+

+ Signifies presence of, and O absence of, virus X in *D. stramonium*.

In Table 5 much the same situation is shown as applied previously with Kentucky 41A. Movement of virus X into the stem tissue of the old plants 1-6 was infrequent, but where it occurred it was associated with the position of the inoculated leaves. In Table 5, plants 1, 2, and 3 were inoculated on basal leaves and plants 4, 5, and 6 on leaves in the upper half of the plant. There is some indication of a greater movement of virus X into stem tissue when leaves on the upper half of the plant are inoculated.

Tables 4 and 5, in conjunction with the previous tables, suggest strongly that this age-induced phenotypic variation in virus X susceptibility is common to *Nicotiana tabacum* varieties irrespective of the genotype. Although genetical experiments have not been made it can be assumed from definite differences in growth habit that the genotypes of Kentucky 41A and Turkish tobacco varieties are also quite different.

VII. TESTS FOR THE PRESENCE OF A VIRUS X INHIBITOR IN THE JUICE FROM LEAVES OF OLD TOBACCO PLANTS

There seemed to be two possible explanations for the phenotypic variation in virus X susceptibility induced by age in tobacco plants. One supposed that the rate of production of inhibitory substances increases with maturity and the

other that the ontogenetic drift in the protein metabolism of aging plants results in changes that restrict the ribose nucleic acid synthesis associated with virus X multiplication. Bawden and Roberts (1947) inoculated plants 5-6 weeks after transplanting with viruses that did not include X and observed that on the same plant young leaves were more susceptible than old, and considered that the differences could be due to a greater accumulation of photosynthetic products in older leaf tissue.

In order to investigate the possibility that the reduced susceptibility of aged plants to virus X was due to a relatively high percentage of inactivating substances, as opposed to a low percentage in young plants, an experiment was planned using the tobacco variety Kentucky 41A, a virulent strain of X in *D. stramonium*, and the quantitative indicator globe amaranth. Two 1 g. lots of *D. stramonium* leaf containing the virulent X strain were taken from the same plant, and one was macerated with 1 g. of leaf from a tobacco plant 14 weeks from transplanting and the other with 1 g. of leaf from a plant that had been transplanted four weeks previously, both lots being made up to 40 ml. with water. Each inoculum was wiped on separate sets of six globe amaranth leaves immediately after mixing, and then again to further sets of six leaves of this indicator after standing for 4, 8, and 16 hours. At each of these three time intervals controls were freshly made from the leaves of old and young tobacco plants as before and inoculated immediately to sets of six globe amaranth leaves. Owing to a lack of sufficient globe amaranth plants, proper randomization of the inoculated leaves could not be obtained, but sufficient comparisons and replications were made to show trends. The results are given in Table 6.

TABLE 6

MEAN NUMBER OF LESIONS ON GLOBE AMARANTH LEAVES FROM INOCULA MADE BY MACERATING X-INFECTED *D. STRAMONIUM* WITH OLD AND YOUNG TOBACCO LEAF RESPECTIVELY

<i>D. stramonium</i> Containing X Mixed with	Mean No. Lesions on Globe Amaranth Leaves from Inoculum			
	Immed. After Mixing	After 4 hr.	After 8 hr.	After 16 hr.
Old tobacco leaf	83	91	62	51
Young tobacco leaf	55	52	43	40

Table 6 gives no indication of an excess of virus X-inactivating substance in old tobacco leaf since the amount of active virus in the inoculum containing it, as judged by the number of lesions on globe amaranth, is greater than in the inoculum made with young leaf. The trend is more in the direction of greater quantities of inactivating substance occurring in young leaf although the steady inactivation with time is more apparent in the inoculum containing old leaf tissue. Further and more detailed experiments would be needed to

discover whether these are real differences or not. The three pairs of controls for the 4, 8, and 16 hour treatments in Table 6 confirmed the finding that, immediately after mixing, the inoculum containing old tobacco leaf gave a greater number of lesions on globe amaranth than that containing young leaf, the mean difference being 20 lesions.

These results suggest that the cause for the reduced susceptibility of tobacco plants to virus X with age is resident in a changed metabolism inhibitory to virus multiplication. It is unlikely that this relationship is dependent on the increasing production of inactivating substances in aging plants.

VIII. DISCUSSION

The biochemical reactions of plants to viruses are complex and require considerable research before they will be understood. However, much can be done by studying the effects of the major controllable factors on plant reaction to viruses. Environmental factors like temperature and light have been studied in this connection by various workers and this paper describes the effect of the non-environmental factor maturity on the interaction between virus X and *N. tabacum*. Such knowledge is an essential basis for research programmes involving studies of the genetics of virus resistance in plants. The testing of progenies in these programmes is often done under relatively uncontrolled conditions. If the factors needing control are known and attention is given to them, masking of the influence of the genotype on the phenotypic reaction to virus is reduced considerably so that genetic ratios can be determined with greater accuracy. The influence of maturity on phenotypic reactions to viruses is not generally realized, and experience has shown the importance of this easily controlled factor in the evaluation of progenies for virus resistance. In some virus-plant combinations, as with virus Y in the potato, the interaction works in the opposite direction to that described in this paper so that more mature plants are needed to accurately assess the inherent resistance of hybrid progeny.

Perhaps the most interesting feature of the results described in this paper is the marked difference in virus status between old plants inoculated at early and late growth stages respectively. Apparently the introduction of virus X into young plants so alters their metabolism that the changes induced in uninoculated plants by aging are inoperative. It may be that the ontogenetic drift in the protein metabolism of tobacco plants inoculated when young is so changed, that the plants are in a continually susceptible condition when compared with old uninoculated plants. If growth is any indication of the rate of virus production in the plants inoculated at an early growth stage, it could be assumed that the rate slows down considerably with age. The points raised pose some interesting biochemical problems, the solution of which could lead to an understanding of what makes plants resistant to virus infection.

IX. ACKNOWLEDGMENTS

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X. REFERENCES

- BALD, J. G., and WHITE, N. H. (1942).—Potato virus X: The average severity of strain mixtures in three varieties of potato. *J. Coun. Sci. Industr. Res. Aust.* **15**: 300-6.
- BAWDEN, F. C., and ROBERTS, F. M. (1947).—The influence of light intensity on the susceptibility of plants to certain viruses. *Ann. Appl. Biol.* **34**: 286-96.
- HUTTON, E. M. (1948).—Some factors affecting localized and systemic necrotic reactions to virus Y in the potato. *Aust. J. Sci. Res. B* **1**: 416-38.
- McKINNEY, H. H., and CLAYTON, E. E. (1945).—Genotype and temperature in relation to symptoms caused in *Nicotiana* by the mosaic virus. *J. Hered.* **36**: 323-31.
- WILKINSON, R. E., and BLODGETT, F. M. (1948).—*Gomphrena globosa*, a useful plant for qualitative and quantitative work with potato virus X. *Phytopathology* **38**: Abstr. p. 28.

AGE AND VIRUS X SUSCEPTIBILITY



An X-infected tobacco leaf with 12 tissue discs, each 4 mm. in diameter, removed for determining the pattern of virus development

THE BACTERIAL FLORA OF AUSTRALIAN FLAX RETTING

By G. W. LANIGAN*

[Manuscript received April 12, 1951]

Summary

An account is given of investigations of both the aerobic and anaerobic bacterial flora associated with Australian flax retting.

Of three media tested, nutrient agar enriched with glucose and yeast extract proved most suitable for aerobic plate counts. While this count varied considerably from ret to ret, its maximum was consistently attained near the end of the first day and at the conclusion of a ret it was at times as little as one-fiftieth of the maximum. Predominant among the aerobes were lactic acid types of *Streptococcus* and species of *Paracolobactrum*. Species of *Flavobacterium*, *Achromobacter*, and the coliform group were present in smaller numbers. Other genera represented in some rets were *Leuconostoc*, *Lactobacillus*, and *Microbacterium*. Maximal presumptive coliform counts of retting liquor varied between 10^2 and 10^8 per ml., 55 per cent. of samples giving counts between 10^4 and 10^6 per ml.

Direct plating of heated suspensions of retted flax on glucose yeast extract agar was the most effective of three methods employed for isolation of the retting bacteria. Four distinct types of retting bacteria (clostridia) were found. Types I and II of these were evidently the predominant retting agents of Australian flax, while types III and IV appeared to play a minor part. Type III retting clostridium was identified as *Clostridium felsineum*, but the other three differ significantly from currently recognized species. Type I is micro-aerophilic; type II is related to the butylic group as exemplified by *Clostridium acetobutylicum*; and type IV is characterized by the formation of a non-diffusible, canary-yellow pigment. Of the non-retting clostridia isolated, 87 per cent. were varieties of *Clostridium butyricum*.

I. INTRODUCTION

The mixed bacterial flora that develops when flax straw is immersed in water at a suitable temperature is derived largely from the soil in which the crop has been grown. In some instances, no doubt, where water from lakes or streams is used, this water may contribute to the bacterial population of the retting tank; but only in exceptional cases would this contribution be appreciable. In the field, bacteria and spores are transferred to the roots by direct contact and to the stems through such agencies as wind and rain. Thus it may be assumed that the composition of the flora is fixed before the straw enters the retting tanks. Doubtless, however, some types of bacteria find conditions in the retting liquor inimical to their development and are soon outgrown, while the proportions of those surviving and multiplying in this environment can be shown to vary with the stage of retting. As would be expected.

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there is present, in addition to the true retting species, a large, mixed, subsidiary flora. Previous investigators in several countries have established that the responsible agents in water retting are spore-forming, anaerobic, or micro-aerophilic bacteria; in modern systems of classification, members of the genus *Clostridium*. The subsidiary flora, on the other hand, comprises species with all degrees of atmospheric requirements (Beijerinck and Van Delden 1904; Störmer 1904; Carbone and Tombolato 1917; Ruschmann and Bavendam 1925; Orla-Jensen and Kluyver 1939; Markova 1940; Allen 1946; Enebo 1947).

The literature relating to the true retting bacteria has been reviewed recently by Enebo (1947) and in a publication by the D.S.I.R., Great Britain (1948), so that no useful purpose would be served here by a repetition of this. However, some of the earlier work will be discussed at a later stage in relation to the present author's findings. Suffice to say that the retting of flax in various localities has been attributed to an organism, or group of organisms, termed variously *Granulobacter* (*Plectridium*, *Clostridium*) *pectinovorum* or to the orange-pigmented *Clostridium felsineum* or to both. The exact characteristics of the former are still difficult to ascertain from the literature, owing possibly to the existence of several varieties or even distinct species with retting ability. Bergey *et al.* (1948) do not accord specific rank to this bacterium but list it as a probable variety of *Clostridium butyricum*. On the other hand, *Cl. felsineum* has been subjected to detailed study and its characteristics are well established (Ruschmann and Bavendam 1925; McCoy and McClung 1935; Bergey *et al.* 1948). The very marked retting activity of the latter organism has been reported by various authors. In 1946, Allen concluded that the bacteria most commonly active in the retting of English flax were strains of *Clostridium tertium*. This appears to be the first occasion on which this clostridium has been found associated with flax retting.

It has been demonstrated (Behrens 1903; Rossi 1904; Rossi and Carbone 1909) that some species of *Bacillus* can ret flax under strongly aerobic conditions, and Allen (1946) isolated an enzyme powder with retting activity from cells of a variety of *B. subtilis*. However, bacteria of this type are believed to play no part in quiescent water retting where anaerobic conditions are soon established.

Relatively little attention has been given to the subsidiary bacterial flora of flax retting. Störmer (1904) identified *Pseudomonas fluorescens*, a coliform bacterium, and yellow-pigmented bacteria, presumably species of *Flavobacterium*, as the principal non-retting types, with yeasts and *Oidium*-like fungi appearing towards the end of the ret. Stutzer (1927) found, *inter alia*, that coliform bacteria and *Streptococcus lactis* were present throughout the ret, while *Bacillus* spp. were detectable in the early stages only and, in 1946, Allen reported essentially similar findings in a study of English flax retting. Enebo (1947), who investigated the flora of Swedish flax rets, found that paracolon bacilli and species of *Flavobacterium* usually predominated, while a *Pseudomonas* species and two species of *Micrococcus* were frequently present in smaller numbers. Apparently Enebo did not encounter lactic streptococci.

In Australia, the large-scale water retting of flax is a comparatively new industry, which developed rapidly in the years following the outbreak of World War II. No previous bacteriological studies of the process in this country have been reported, although Jensen (1941) published an account of his investigations of the fungal flora associated with dew retting, which has been practised in some of our flax-growing districts until quite recently. It is the purpose of the present paper to record the results of studies in the bacteriology of local flax retting carried out by the author during the past four years. The subsidiary flora as well as the retting bacteria have been investigated. Investigation of the aerobic and facultative species was carried out and is described first; this approach appearing logical in view of the prior development of these bacteria in the actual retting process.

II. THE AEROBIC AND FACULTATIVELY ANAEROBIC FLORA

(a) *Materials and Methods*

(i) *Aerobic Plate Counts*.—Laboratory tank rets, employing the standard "Belgian" type of schedule, were the source of material for enumeration and isolation of the aerobic species of bacteria present in flax retting liquor. These rets utilized a variety of flax crops grown in different districts. Standard nutrient agar was found to be unsuitable for aerobic plate counts, as many of the colonies that developed on this medium were too small for convenient counting. Allen (1946) reported that nutrient agar gave lower counts than either his carrot extract agar or potato agar. Three media were investigated in the present study; namely, a glucose yeast extract agar (G.Y.A.), a potato extract agar (P.A.), and Allen's (personal communication) carrot extract agar (C.A.).

For the counts, samples of liquor were withdrawn aseptically from a number of laboratory rets at intervals of 24 hr. throughout the retting period. In each case, liquor was taken from three levels in the tank and a composite sample prepared by mixing in a shaking machine. Serial dilutions of these samples to 1 in 10^7 were prepared in sterile distilled water. Duplicate counts were made with each of the three media described above. Incubation was at 30°C.

(ii) *Isolation of Aerobic and Facultative Species*.—Several of each type of colony present were picked from the plate cultures used for the aerobic counts and replated twice to ensure purity of the strains. Stock cultures were preserved under sterile liquid paraffin on either nutrient agar or glucose yeast agar, according to individual requirements.

(iii) *The Coliform Flora*.—In addition to observations regarding these organisms made in the general study of aerobic species, a specific investigation of the coliform flora was carried out. Presumptive coliform counts, using MacConkey's neutral red, lactose, bile salt, peptone water, were made on samples of liquor from 18 laboratory and 11 mill rets. The mill samples were transported to the laboratory packed in ice. The cultures from the highest and second highest dilutions, showing acid and gas formation within 48 hr. at 37°C.,

were plated out on MacConkey agar. After incubation at 37°C. for 24 hr., representative colonies were picked off and replated to ensure purity. The pure cultures were submitted to the standard tests employed for identification and typing according to the scheme of Wilson *et al.* (1935).

TABLE 1
AEROBIC PLATE COUNTS OF BACTERIA IN FLAX RETTING LIQUORS

Ret No.	Retting Time (hr.)	Count (millions per ml.)		
		G.Y.A. ¹	P.A. ²	C.A. ³
1	24	43.0	—	44.0
	24	85.0	79.5	86.5
2	48	9.4	9.3	9.7
	72	7.5	6.9	7.0
	96	5.2	5.3	—
3	24	370.0	347.0	348.0
	48	39.0	39.0	34.0
	72	35.7	29.8	32.0
	96	10.4	7.6	—
4	24	164.5	175.0	159.0
	48	6.6	2.7	7.0
5	24	27.6	22.8	19.0
	48	16.3	14.9	11.7
	72	11.2	10.7	5.1
	96	5.0	4.05	1.95
6	24	84.5	94.5	90.0
	48	34.0	33.0	38.5
	72	11.0	11.0	9.5
	96	1.8	2.0	2.2
7	24	61.0	55.0	51.0
	48	49.0	51.5	45.0
	72	13.0	8.0	6.5
	96	3.0	1.4	3.0

¹ G.Y.A., glucose yeast extract agar.

² P.A., potato extract agar.

³ C.A., carrot extract agar.

(b) Experimental and Results

(i) *Enumeration of Aerobic Flora.*—Table 1 summarizes the results of aerobic plate counts on samples of liquor from several laboratory rets. The figures given are means of duplicate determinations. Statistical analyses of these results were carried out and the significance of deviations was determined by use of the χ^2 test, with appropriate corrections for continuity. In the examination of variation between duplicate results, the values of χ^2 were found to be in good agreement with expectation, thereby indicating that the method was of satisfactory accuracy. The effect of medium on the count was significant at

the 1 per cent. level in approximately one-half of the tests. The media, in descending order of count were, generally, G.Y.A., P.A., and C.A., the counts being evenly spaced at 24 and 48 hours, but at 72 and 96 hours G.Y.A. and P.A. were close together. Highly significant interactions of medium and dilution occurred in two instances but in neither were the above conclusions affected.

Although G.Y.A. usually gave slightly higher counts than did P.A., it was observed that colonies were frequently better developed and any pigmentation was more marked on the latter medium than the former. It would seem therefore that P.A. may be better suited to some of the bacterial types present in retting liquor.

At first, colony counts were made after incubation of plates for 2, 3, 4, 5, and 6 days. These early tests indicated that 3-4 days was the most suitable incubation period. At this stage, colonies were of a convenient size for counting and did not increase in numbers on further incubation. Moreover, with more prolonged incubation, colonies of micro-aerophilic species usually appeared and rendered counting uncertain, since some of these approximated in size the smaller aerobic colonies. Towards the end of a ret, aerobic bacterial plate counts were frequently unreliable, owing to the development of large numbers of yeast colonies.

By reference to Table 1, it will be seen that, after 24 hours retting, the aerobic plate count is higher than at any subsequent stage, and it therefore appears that this count reaches its maximum at or about the end of the first day of retting. In the rets studied here, it will be seen that the maximal aerobic plate count varies considerably; viz. from 27.6 to 370×10^6 per ml., and at the end of the retting period the count may be as low as one-fiftieth of its maximum, the rate and extent of this decline varying from ret to ret also.

(ii) *Composition of the Aerobic Flora*.—Detailed study of 183 cultures showed that they comprised the following genera, the percentage of the total belonging to each genus being shown in parentheses: *Streptococcus* (35.0), *Paracolobactrum* (34.0), *Flavobacterium* (9.8), *Achromobacter* (6.6), *Lactobacillus* (4.9), *Aerobacter* (4.4), *Leuconostoc* (3.3), *Microbacterium* (1.0), *Escherichia* (0.5), and *Bacillus* (0.5).

In the following paragraphs, details of the morphological and cultural characteristics of the various bacterial groups encountered are given. In most cases, specific identification has been achieved but in others this has not been possible. For the purpose of placing them on record, those organisms not conforming in their properties to recognized species are described in some detail.

Streptococcus.—Morphologically, the cultures classified as members of this genus were Gram-positive, spherical to ovoid diplococci, with little tendency to form chains of more than four to six cells, even in liquid media. They were identified as *Streptococcus lactis* (Lister) Löhnis, being differentiated from *Strep. faecalis* on the one hand and *Strep. cremoris* on the other by the following properties: growth at 40° but not at 45°C., and growth in the presence of 4 per cent. but not 6.5 per cent. sodium chloride.

Leuconostoc.—The six cultures tentatively allotted to this genus differed from the other streptococci in that they produced little or no change in litmus milk medium. In this respect and in view of the marked stimulation of their growth by yeast extract, they certainly resemble described species of *Leuconostoc* (Hucker and Pederson 1930; Bergey *et al.* 1948). On the other hand, none of the strains isolated showed evidence of polysaccharide gum formation in sugar media. Moreover, they did not correspond in their reactions to any of the species listed by Bergey *et al.* (1948). In view of the small numbers found, however, these cultures have not been investigated further.

Paracolobactrum.—Cultures classified as members of this genus had the general characteristics of the coliform group of bacteria but exhibited a delayed or incomplete fermentation of lactose. In lactose peptone water, acid formation was generally evident in 2-5 days and was usually accompanied by a small bubble of gas in the Durham tube, which did not increase appreciably on prolonged incubation. In litmus milk medium, acid only or acid with slow coagulation occurred. Apart from their slow or incomplete fermentation of lactose, 57 of the cultures of this type had the characteristic properties of the *aerogenes-cloacae* group of bacteria. Most strains liquefied gelatin within 2 weeks at 22°C. Indole production was variable. Sixteen strains formed a golden pigment of varying intensity on nutrient agar. Consequently, it was thought at first that they may be related to the plant pathogens, *Erwinia carotovora* and *E. erivanensis*; however, they did not exhibit any digestive action on sterile, raw carrot or potato. Thus these strains must be regarded as pigmented varieties of paracolons. The observed characteristics of the cultures in this group identify them as *Paracolobactrum aerogenoides* Borman, Stuart, and Wheeler (1944).

The remaining five cultures of paracolon bacilli, while utilizing citrates as a sole source of carbon, were positive in the methyl red test, did not form acetyl methyl carbinol, and did not liquefy gelatin. Accordingly, they were identified as *Paracolobactrum intermedium* Borman, Stuart, and Wheeler (1944).

Flavobacterium.—Eighteen cultures were identified as members of the genus *Flavobacterium*. Three of these were *Flavobacterium estero-aromaticum* (Omeliński) Bergey *et al.* (1948). The remaining cultures represented six types, but none could be definitely identified with the species described by Bergey *et al.* (1948). The principal features of these unidentified strains are given in Table 2.

Achromobacter.—None of the cultures classified as members of this genus could be identified with species described by Bergey *et al.* (1948). The properties of the 12 strains studied are recorded in Table 2.

Aerobacter.—Of the eight cultures isolated that belonged to this genus, six were *Aerobacter aerogenes* (Kruse) Beijerinck and two were *Aerobacter cloacae* (Jordan) Bergey *et al.* (1948). On the basis of Wilson's (1935) classification, four of the *A. aerogenes* strains were type I and two were type II.

TABLE 2
CHARACTERISTICS OF ORGANISMS CLASSIFIED AS SPECIES OF *ACHROMOBACTER* AND
FLAVOBACTERIUM

Genus	No. of Cultures	Pigment on Agar	Motility	Gelatin Liquefaction	Litmus Milk	Nitrate Reduction	Glucose	Maltose	Sucrose	Lactose	Mannitol
<i>Achromobacter</i>	11	None	+	Not liquefied	Rennett C, slowly peptonized	Reduced to nitrite	A	O	A slow	O	O
	1	None	+	Stratiform; slow	Rennett C, slow. Not peptonized	Not reduced	A	O	A	O	A
<i>Flavobacterium</i>	9	Pale golden	+	Saccate; 3-4 weeks	A, C slow	Reduced to nitrite	A	A	O	A slow	A
	2	Pale golden	+	Infundib.; 5-12 days	A, C slow	Reduced to nitrite	A	A	A	A	A
	1	Pale golden	+	Infundib.; 5-12 days	Rennett C, peptonized	Reduced to nitrite	A	A	A	O	A
	1	Pale golden	—	Not liquefied	A, slight	Reduced to nitrite	A	A	O	O	A
	1	Pale yellow	—	Stratiform; slow	A, slight	Reduced to nitrite	A	A	A	A	O
	1	Yellow	+	Stratiform; slow	A	Reduced to nitrite	O	O	O	O	O

Gelatin incubated at 22°C., all other cultures at 30°C. A = acid; C = coagulation; O = no change.
None of the cultures produced indole.

Escherichia.—The sole representative of this genus isolated was identified as *Escherichia intermedium* (Werkman and Gillen) Vaugh and Levine. It belonged to Wilson's type 1.

Lactobacillus and *Microbacterium*.—Table 3 summarizes the characteristics of the 11 cultures identified as members of these genera. Apart from generic allocation, it has not been possible to identify any of them with described species. Their infrequent occurrence in flax retting liquor has not warranted further investigation.

TABLE 3
CULTURAL CHARACTERISTICS OF THE GRAM-POSITIVE, NON-MOTILE, NON-SPORING
BACILLI ISOLATED

No. of Cultures	Litmus Milk	Catalase	Nitrate Reduction	Glucose	Maltose	Sucrose	Lactose	Mannitol	Generic Classification
5	O or sl. A	—	Not reduced	A	A	A	O	O	<i>Lactobacillus</i>
4	A, C	—	Not reduced	A	A	O	A	O	<i>Lactobacillus</i>
2	A with slow C	+	Reduced to nitrite	A	O	O	A	O	<i>Microbacterium</i>

Other features common to the three groups were aerobic and facultatively anaerobic growth; absence of motility; and no liquefaction of gelatin. A = acid; C = coagulation; O = no change.

Bacillus.—Only one culture belonging to this genus was isolated. Its characteristics indicated that it was a variety of *Bacillus megatherium* De Bary.

(iii) *The Coliform Flora*.—In the investigation of the aerobic flora of flax retting liquor described above, only nine cultures belonging to the coliform group of bacteria were isolated. Thus their proportion of the total cultures studied; viz. 5 per cent., would not indicate that they constituted a very significant part of the aerobic species present. However, in view of the importance attached to the presence of coliform bacteria when assessing the pollutional effect of retting effluent on streams etc., it was decided to study a wider range of retting liquors, using more specific methods for the detection of these bacteria. Accordingly, presumptive coliform counts were made on a further 29 samples of retting liquor; 18 from laboratory rets and 11 from mill rets. Figure 1 shows the percentage distribution of these counts. All counts were made after 20-24 hr. retting, i.e. when the aerobic plate count was at or near its maximum.

It will be seen from Figure 1 that the 24-hr. presumptive coliform count is subject to wide variations from ret to ret. Fifty-five per cent. of the counts were found to lie between 10^4 and 10^6 per ml., while the remainder were fairly evenly distributed above and below these limits, the total range of the counts being from 10^2 to 10^8 per ml. The fact that only 24 per cent. of the liquor

samples gave counts higher than 10^6 per ml. probably explains why so few coliform bacteria were detected in the study of the aerobic flora, based on cultures used for aerobic plate counts, which ranged from 30 to 300×10^6 per ml.

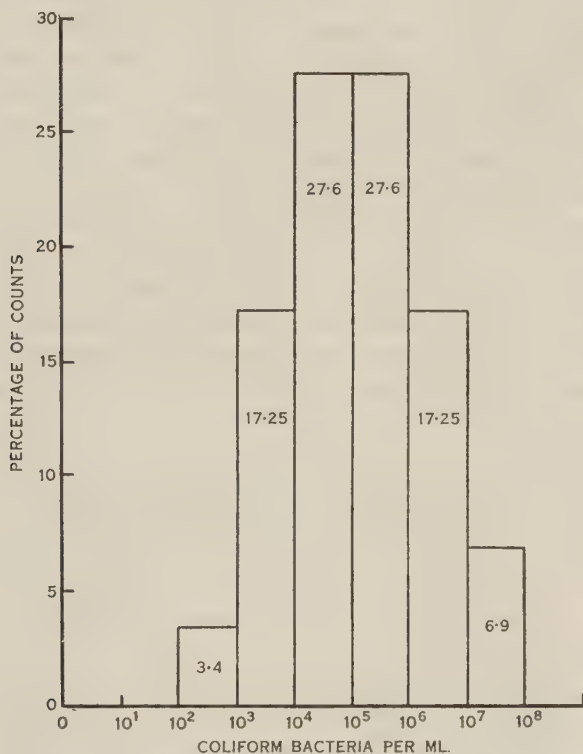


Fig. 1.—Percentage distribution of presumptive coliform counts.

By plating and selection of discrete colonies, 110 bacterial strains were isolated from the presumptive coliform count tubes. As would be expected, however, it was found that, in a number of instances, duplicate cultures of the same coliform type had been recovered from the one ret. For this reason 21 cultures were discarded. One other culture proved to be a species of *Proteus* and was also discarded. Ultimately, therefore, 88 cultures remained to be classified and, of these, 26 were shown to be paracolon types by their delayed or incomplete fermentation of lactose. The coliform and paracolon types found and the percentage of the total belonging to each type are shown in Table 4.

Identification of the various coliform types was based on the classification of Wilson *et al.* (1935) and that of the paracolon species was in accordance with descriptions given in Bergey's Manual (1948).

III. THE ANAEROBIC AND MICRO-AEROPHILIC FLORA

(a) *Materials and Methods*

In order that the investigation of the anaerobic flora of Australian flax retting, particularly the retting species, might be reasonably comprehensive, 41 flax samples were obtained from all of the mills employing water retting. Moreover, where possible, crops were selected that had been grown in localities representative of the total supply of flax straw to the particular mill. Samples were obtained of six crops from each of the six Victorian mills, viz. Ballarat, Colac, Drouin, Lake Bolac, Myrtleford, and Strathkellar; of two crops from the Hagley mill in Tasmania; and of three crops from the Boyup Brook mill in Western Australia. All samples were retted in the laboratory, using the Belgian type of anaerobic retting schedule in current use in Australia. At the completion of retting, lengths of straw were withdrawn aseptically and at random from each sample and allowed to drain for a short time in a sterile tube. After being cut into lengths of approximately $\frac{1}{4}$ in., 5 g. of the retted straw samples were shaken mechanically with glass beads in 95 ml. of sterile distilled water, to obtain an even distribution of the bacteria present. Serial dilutions of these primary suspensions were prepared and used for the isolation of anaerobic bacteria as described below.

TABLE 4
ISOLATIONS FROM COLIFORM COUNT TUBES

Type	Percentage
<i>Esch. coli</i> type I	11.4
<i>Esch. intermedium</i> type I	17.1
<i>Esch. intermedium</i> type II	5.7
<i>Aerobacter aerogenes</i> type I	17.1
<i>Aerobacter aerogenes</i> type II	1.1
<i>Aerobacter cloacae</i>	8.0
Irregular type II	3.4
Irregular type IV	2.3
Irregular type VI	4.6
<i>Paracol. coliforme</i>	1.1
<i>Paracol. intermedium</i>	3.4
<i>Paracol. aerogenoides</i>	25.0

As this study was to be limited to the spore-forming anaerobic species present, all suspensions of retted straw were heated in a water-bath for 20 min. at 80°C. to destroy vegetative cells, in particular those of the facultative anaerobes. Three different methods were used for isolation of clostridial species from the heated suspensions of retted straw; namely:

(A) Incubation at 37°C. in a glucose potato mash medium* for 7 days after fermentation commenced, followed by heating for 20 min. at 80°C., then plating out on G.Y.A.;

* As described in "Manual of methods for pure culture study of bacteria." Leaflet No. 2, p. 22. Biotech. Publications, Geneva, N.Y., 1944.

(B) Incubation at 37°C. in the raw potato broth of Allen (1946) for 7-10 days, followed by heating to destroy vegetative cells and plating out of the tubes from the highest dilutions producing softening of the potato;

(C) Direct plating of the retted straw suspension on G.Y.A.

In all three methods, representative colonies were picked from the plates into glucose potato mash, and after a short period of incubation, i.e. when fermentation was obvious, the cultures were replated. Usually, three platings were needed to ensure purity. Each strain recovered was tested for freedom from aerobic contaminants. Unless otherwise stated, all cultures of anaerobic organisms were incubated in specially made jars, in an atmosphere of hydrogen and nitrogen plus 5 per cent. CO₂, the oxygen being removed by means of a palladium-asbestos catalyst — Wright's (1943) cold capsule as described by Hayward (1945).

Allen's (1946) raw potato medium was modified by substituting yeast-peptone water for the nutrient broth used by him, as better growth of most cultures isolated was thereby obtained. Growth of these anaerobes in the raw potato medium from small inocula, such as high dilutions of retted straw, was rendered much more certain by simultaneous inoculation with a non-retting aerobic organism, such as *Aerobacter aerogenes*, or one of the paracolon species described in the section relating to the aerobic flora. This technique obviated the problem of dissolved oxygen, which could not be removed by the usual process of heating and, in addition, it permitted incubation without resort to special anaerobic jars. The cells of the aerobe were, of course, readily destroyed by subsequent "pasteurization."

Methods (A) and (B) were used for straw samples from Colac, Hagley, Lake Bolac, and Strathkellar; methods (B) and (C) were used for the Ballarat, Drouin, and Western Australian samples; and method (C) only was employed in the study of the samples from the Myrtleford district.

The ability, or otherwise, of the various anaerobic cultures to ret flax was determined by the method of Allen (1946). This method employed a medium prepared by sterilizing air-dry flax straw in tubes at 15 lb. steam pressure for 20 min. and subsequently covering the straw with sterile water. It was found, however, that yeast water gave better results than tap water and this has therefore been used throughout this investigation.

After a suitable period of incubation to test sterility, the sterile flax medium was usually inoculated with 1 ml. of an actively growing potato mash culture of the various strains of clostridia. Some of the organisms, however, did not grow well in potato mash and, in such cases, glucose yeast peptone water cultures were used as inoculum. The cultures were incubated at 37°C. until the straw was retted, or for a maximum of 10 days. As with the raw potato broth cultures, simultaneous inoculation with a non-retting facultative anaerobe, such as *A. aerogenes*, and "aerobic" incubation was usually superior to incubation in an anaerobic jar. It was realized that objection to this practice could be raised, on the grounds of symbiotic effects resulting in a retting action which might not be evident in pure cultures. Therefore, all strains were

tested both in pure culture and in association with the facultative anaerobe. None of the organisms studied showed evidence of retting in the latter cultures without it being shown in the former also.

However, almost invariably, retting proceeded more rapidly in the mixed cultures. As Allen (1946) has pointed out, the use of heat-sterilized flax for determining the retting ability of a particular organism is open to the objection that sterilization produces some physical and possibly chemical changes in the straw, which could lead to false positive results. Experience has shown, however, that this test does give a clear-cut differentiation of retting and non-retting types of bacteria. While there were obvious differences in the rate and completeness of retting by different strains, the changes produced in the flax by retting types were profoundly different from those occurring in the presence of non-retting organisms, such as members of the aerobic flora. A very few anaerobic organisms were isolated, however, which did not conform to any of the main retting groups encountered, and which appeared capable of bringing about partial retting of the sterilized flax. It has not yet been possible to determine whether these few strains would have a similar effect on unheated flax and, in any event, it seems most unlikely that they play a part of any great importance in normal retting.

(b) *Experimental and Results*

(i) *Isolation*.—Initially, 535 cultures of spore-forming anaerobic bacteria were isolated and purified by the methods outlined above. These were submitted to a preliminary screening based on morphology, colonial characteristics, and action on sterilized flax. As a result of this screening, approximately half of the cultures isolated from each ret were discarded as obvious replications, while, wherever possible, duplicate or triplicate strains of each type found were retained. Ultimately, 293 cultures were submitted to detailed investigation.

Table 5 shows the proportions of retting and non-retting members of the genus *Clostridium* isolated by each of the three methods employed. Unfortunately, because of overlapping of work with flax from different sources, the complete unsuitability of method A did not become apparent until cultures from a considerable number of rets had been studied in some detail. This method was then immediately discarded. With regard to methods B and C, it will be seen from Table 5 that the latter yields twice the proportion of retting organisms obtainable by the former and is therefore the method of choice. It is of interest to note that, even with the direct plating of method C, equal numbers of retting and non-retting types of sporing anaerobes were isolated.

(ii) *The Retting Flora*.—The observed characteristics of the retting strains of sporing anaerobes isolated showed that they constituted four distinct, homogeneous groups labelled for convenience types I-IV. The members of three of these groups (I, II, and IV) had properties that clearly differentiated them from any of the species described by Bergey *et al.* (1948), but group III was readily identified as *Clostridium felsineum* (Carbone and Tombolato) Bergey *et al.* (1948). Retting clostridium type I, which was micro-aerophilic, appeared

similar to the *Plectridium pectinovorum* of Störmer (1904) and the plectridial types described by Weizmann and Hellinger (1940). Its relationship to these organisms and significant points of difference will be discussed later.

TABLE 5
EFFECT OF ISOLATION METHOD ON PROPORTION OF RETTING AND NON-RETTING CLOSTRIDIA

Origin of Flax	Method A		Method B		Method C	
	Retting	Non-Retting	Retting	Non-Retting	Retting	Non-Retting
Colac	6	39	11	24	—	—
Hagley	0	9	2	4	—	—
Lake Bolac	0	46	14	41	—	—
Strathkellar	0	32	2	26	—	—
Ballarat	—	—	8	26	24	16
Drouin	—	—	7	27	36	35
Boyup Brook	—	—	9	12	14	11
Myrtleford	—	—	—	—	22	33
Totals	6	126	53	160	95	95
	(4.5%)	(95.5%)	(24.8%)	(75.2%)	(50.0%)	(50.0%)

The retting activity of clostridium type I was less marked than that of the other three types. Generally, retting was incomplete and a few strains did little more than soften and loosen the cortical and epidermal tissues. Morphological features of retting clostridium type I are shown in Plate 1, Figures 9 and 10, and typical colonies are illustrated in Plate 1, Figures 1-3. All strains of *Cl. felsineum* isolated proved to be very active retting agents; in fact, by far the most active of the four types encountered. None of the author's strains of *Cl. felsineum* fermented inulin and none could be shown to reduce nitrates, even at 10 p.p.m. KNO_3 , although all reduced 1000 p.p.m. of nitrite to ammonia. Thus, in the first two properties they differed from the description of Bergey *et al.* (1948). It is perhaps worthy of note that one of Carbone's original strains, possessed by the author, could only reduce 10 p.p.m. of KNO_3 and also failed to ferment inulin. Consequently, it would appear that nitrate reduction and inulin fermentation are variable properties of *Cl. felsineum*. Photomicrographs of a local strain of this organism are shown in Plate 1, Figures 13 and 14, for comparison with the other types.

The characteristics of the Australian retting clostridia types II and IV, which do not conform to descriptions of recognized clostridial species, are given in detail in the following paragraphs. All cultures were grown anaerobically at 37°C. and morphological descriptions are based on Gram-stained smears from glucose yeast agar stroke cultures.

(iii) *Retting Clostridium Type II*.—Twenty strains of this type were studied.

Morphology and staining, medium size rods with markedly incomplete fission at 24 hours; but at 48 hr. the chains and filaments have largely disappeared. Rods are 0.6-0.8 μ diam. and mostly 2-7 μ long; axis straight to slightly curved, ends rounded, sides parallel; motile; Gram-positive when young.

Sporulation occurs early and freely. The body of the sporangium is only slightly greater in diameter than the vegetative cells but it is distended by an elliptical spore, which arises subterminally, although in the majority of mature sporangia the spore appears terminal. Sporangial cells are usually slightly curved, some appreciably so. Their length varies between 5 and 12 μ , with the majority 6-9 μ . Free spores are elliptical in outline, their dimensions varying between 1.6 and 2.4 μ in length and 1.0 and 1.2 μ in width; most are 1.8-2.0 $\mu \times 1.1 \mu$ (Plate 1, Figs. 11 and 12).

Glucose gelatin, liquefied within 7-21 days.

Nutrient agar slope, little or no growth.

Nutrient broth, at most, slight deposit.

Glucose yeast agar surface colonies, characteristic colony irregular in outline, with a "woolly" or myceloid margin. In 3 days on dry plates, they are 2-3 mm. diam., but on even slightly moist media there is a marked tendency to spread, forming effuse, amoeboid projections. The colony is differentiated into a circular, low-convex, opaque, greyish white, central knob, and an effuse, translucent marginal zone. The centre is smooth, glistening, and butyrous, while the effuse portion is largely embedded in the agar. This penetration of the medium is myceloid in appearance. The relative sizes of the convex centre and the effuse margin vary from strain to strain. Occasionally, the central, convex zone is unusually well developed and slightly viscid in consistency, while the effuse, myceloid margin is quite narrow or may be almost non-existent. It is considered that this type of colony may represent a mucoid phase (Plate 1, Figs. 4-6).

Glucose yeast agar deep colonies, in 3 days, approximately spherical, "woolly" balls, 1 mm. diam.; medium shattered by gas.

Glucose yeast agar stroke culture, abundant growth, filiform on dry slopes but spreading with finger-like projections if medium is moist; slightly raised, smooth, glistening, butyrous centre; effuse, matt margin with myceloid penetration of the agar; "woolly" periphery.

Glucose yeast peptone water, heavy turbidity and heavy amorphous deposit; much gas.

Litmus milk, acid, reduction, gas, and clot; frequently a stormy coagulation; no digestion of the curd.

Potato mash, active fermentation with "head"; complete diastatic action.

Cooked brain medium, some gas; no blackening or digestion.

Coagulated egg albumin, no visible effect but, after several days, slight softening is usually detected by probing.

Indole, not formed.

Hydrogen sulphide, small to moderate amounts produced on glucose yeast agar in 3-7 days.

Nitrates, not reduced.

Nitrites, reduced, presumably to ammonia, in 2-3 days.

Carbohydrates and related compounds, acid and gas produced from glucose, galactose, maltose, sucrose, lactose, starch, and pectin. Inulin, mannitol, glycerol, and calcium lactate not fermented. Cultures in glucose yeast peptone water have a distinct odour of butanol.

Sterilized flax in yeast water, active retting by all strains.

Raw potato in yeast water, rapid disintegration.

Optimum temperature, not determined; grows well at 30° and 37°C.

Atmospheric requirements, obligate anaerobe.

Distinctive features, colony on glucose yeast agar; requires fermentable carbohydrate for growth; ability to ret flax.

Habitat, presumably soil.

(iv) *Retting Clostridium Type IV*.—Three strains of this type were studied.

Morphology, slender rods, $0.4\ \mu$ diam. \times $2\text{--}7\ \mu$ long, most $3\text{--}4\ \mu$; occurring singly, in pairs end to end and in small clusters with some palisade formation, some short filaments; axis straight to slightly curved, ends rounded, sides parallel; motile; Gram-positive when young. Sporulation occurs early and freely. The cell is distended at sporulation by an elongated, subterminal spore with a pronounced terminal "cap." The spore frequently occupies half the length of the sporangium, which is usually curved and often shows a definite kink corresponding in position to the junction of the spore and the cytoplasm. Sporangial length is fairly uniform, most cells being $6\text{--}7\ \mu$ long with a maximum range of variation from 5 to $8\ \mu$. The free spores are long and narrow, elliptical or bean-shaped in outline, $2.2\text{--}3.0\ \mu$ long \times $0.7\text{--}0.9\ \mu$ wide, most being $2.5\text{--}2.7\ \mu \times 0.8\ \mu$ (Plate 1, Figs. 15 and 16).

Glucose gelatin, liquefied in 7-11 days.

Nutrient agar, no growth.

Nutrient broth, no growth.

Glucose yeast agar surface colonies, in 3 days, circular, 1-1.5 mm., entire, low-convex or umbilicate, smooth and glistening, opaque, canary-yellow by reflected light, viscid consistency (Plate 1, Figs. 7 and 8).

Glucose yeast agar deep colonies, biconvex discs, 1 mm. diam. in 3 days, canary-yellow colour, medium disrupted by gas.

Glucose yeast agar stroke culture, good, filiform growth; smooth and glistening surface; canary-yellow, non-diffusible pigment; soft butyrous to viscid consistency.

Glucose yeast peptone water, heavy uniform turbidity with much gas, pale yellow viscid deposit.

Litmus milk, acid, gas, reduction, coagulation; usually a stormy clot within 2-4 days; no digestion of curd.

Potato mash, active fermentation with "head," pale canary-yellow pigment, complete diastatic action.

Cooked brain medium, slight gas, no blackening or digestion.

Coagulated egg albumin, no visible change but after several days softening can be detected by probing.

Indole, not formed.

Hydrogen sulphide, traces formed on glucose yeast agar.

Nitrates, not reduced.

Nitrites, 0.1 per cent. KNO_2 reduced, presumably to ammonia.

Carbohydrates and related compounds, acid and gas produced from glucose, galactose, maltose, sucrose, lactose, starch, and pectin. Inulin, mannitol, glycerol, and calcium lactate not fermented. Cultures in glucose yeast peptone water have marked odour of butanol.

Sterilized flax straw in yeast water, active retting; this organism appears to possess retting ability intermediate between that of *Cl. felsineum* and type II.

Raw potato in yeast water, rapid disintegration.

Optimum temperature, not determined; grows well at 37°C . and well but more slowly at 30°C .

Atmospheric requirements, strictly anaerobic.

Distinctive features, formation of a non-diffusible canary-yellow pigment; morphology of sporangia; active retting of flax; no growth in the absence of a fermentable carbohydrate.

Habitat, probably soil.

(v) *Relative Importance of the Four Retting Clostridia*.—In view of the observed differences in retting activity of the organisms encountered in this investigation, it was obviously desirable to establish, if possible, the relative frequencies with which each type occurred in Australian flax retting. Retting clostridia types I, II, III, and IV, respectively, were found in approximately $\frac{3}{4}$, $\frac{1}{2}$, $\frac{1}{3}$, and $\frac{1}{10}$ of the rets studied. However, it was shown in Table 5

that marked differences existed in the numbers of retting organisms recovered by the three methods employed and, consequently, these overall figures do not give a true picture of the respective frequencies with which the four types of retting clostridia occur. In view of this deficiency, the numbers and percentages of the four types isolated by each method have been determined and these figures are presented in Table 6.

TABLE 6
EFFECT OF ISOLATION METHOD ON RECOVERY OF THE FOUR TYPES OF
RETTING CLOSTRIDIA

Method of Isolation	No. of Rets	No. of Rets from which Isolated			
		Type I	Type II	Type III	Type IV
A	18	3 16.7%	0	0	0
B	33	16 48.5%	4 12.1%	1 3.0%	2 6.1%
C	21	15 71.5%	14 66.7%	10 47.6%	1 4.8%

It is evident from Table 6 that only the results obtained by method C are of real value in determining the relative importance of the various types of retting organisms in Australian flax retting. From these findings it appears that clostridial types I and II are equally prominent in our retting, while type III is less commonly present. So few cultures of type IV were isolated that it is apparent that this organism plays little part in flax retting in this country. The dominant role of types I and II and the minor activity of type III become even more evident from a consideration of the following three observations. In all rets from which retting bacteria were isolated, either type I or type II was found. In 43 per cent. of the rets for which method C was used, both of these organisms were present in similar numbers. On the other hand, retting clostridium type III was never found alone and, in fact, only an odd colony of this organism was usually found on the plates. It might be argued, of course, that the method of isolation was not well suited to type III clostridium and that this was responsible for the relative paucity of its colonies. That this is not so is shown by the fact that, with some Belgian flax examined and in some rets inoculated with this organism, its colonies were well developed and predominant on the plates. It is therefore evident that clostridial types I and II are the agents most commonly responsible for flax retting in Australia and that, in about half of the rets, both types are present in approximately equal numbers. The significance of these findings will be discussed in a later section of this paper.

(vi) *The Non-retting Anaerobic Flora*.—Of 381 cultures of non-retting clostridia initially isolated, 174 were studied in detail, the remainder being discarded as obvious replications after the preliminary screening. On the basis

of morphological and cultural characteristics, these non-retting clostridia were separable into six groups.

Groups I, II, and III, comprising 87 per cent. of the cultures studied, had morphological and cultural properties that identified them with the *Clostridium butyricum* group (Bergey *et al.* 1948). Group I differed from group II in failing to reduce nitrates, although both reduced nitrites to ammonia. Thus these groups were identified as varieties of *Cl. butyricum* Prazmowski and *Cl. multifementans* Bergey *et al.*, respectively. It is noteworthy, however, that both groups of organisms showed a wider range of fermentative activity than the type species, in their consistent fermentation of mannitol, glycerol, and calcium lactate. On the other hand, none of the strains produced the typical "stormy" fermentation of milk. The single culture of group III did not ferment starch, inulin, mannitol, glycerol, or lactate and failed to reduce nitrates. Thus it was identified as *Cl. beijerinckii* Donker.

The colonial morphology of these butyric clostridia was subject to appreciable variation from strain to strain. As a considerable number of strains have been studied here and the colony types found may therefore be deemed representative of those likely to be encountered, descriptions and percentages of the various forms found in groups I and II are shown in Table 7.

TABLE 7
PERCENTAGES OF VARIOUS COLONY FORMS IN BUTYRICUM-TYPE CLOSTRIDIA

Description of Colony	Percentage of Cultures	
	Group I	Group II
A. Circular, entire, smooth, glistening, low-convex, opaque to translucent, creamy white; typical colony	47	59
B. Body of colony as for A but with translucent peripheral buds or effuse, ragged outgrowths	5	33
C. Slightly irregular, slightly umbonate, opaque centre, translucent spangled margin	32	8
D. Circular, pulvinate, collapsing later, greyish white, viscid, semi-fluid; mucoid variant	13	0
E. As for A, but pale mauve pigment formed when first isolated	3	0

It will be seen from Table 7 that approximately half of the strains in both groups formed the typical colony of *Cl. butyricum* (colony type A). The several variant colonies, however, tended to be more common in one or other of the two groups. Thus, colony type B was found predominantly in group II, while types C, D, and E were most commonly formed by group I, types D and E, in fact, being confined to the latter group.

None of the other non-retting clostridia, groups IV-VI, could be identified with currently recognized species and, as relatively few cultures of each were isolated, indicating that they were adventitious types of no significance to the retting process, they have not been studied further.

IV. DISCUSSION AND CONCLUSIONS

(a) The Aerobic and Facultatively Anaerobic Flora

The aerobic plate count of liquor from anaerobic rets with Australian flax has been found to reach its maximum at or about the end of the first day. From then on, the count steadily declines and, towards the end of the ret, may fall to as little as one-fiftieth of its maximum. The maximal count varied appreciably from ret to ret. In the rets studied here, the total range of variation was from 26 to 370×10^6 per ml. The results obtained in the present study are in good agreement with those of Allen (1946) in England, who reported counts of 20 to 60×10^6 per ml. at 20 hr., falling to about 5×10^5 per ml. at 65 hr., in anaerobic rets, while the liquor from aerated rets gave much higher counts which remained high throughout the ret. Allen also found that counts per gram of moist straw were somewhat higher than in the liquor.

All of the aerobic bacteria isolated from retting liquor in this investigation are representative of types commonly found in soil and water and on plants. Thus, their presence in flax rets might almost have been predicted. As far as is known at present, none of the aerobic species encountered is able to ret flax. None of the strains tested had any apparent effect on flax sterilized in the autoclave. On the other hand, the lactic streptococci, the paracolons, and the coliforms, which constitute the majority of the aerobic flora, are active fermenters of the simpler carbohydrates and related substances. Consequently, by their action on the soluble constituents of the flax stem, they may be expected to contribute appreciably to the total acidity of retting liquor. Thus, the importance of giving the flax straw a preliminary rinse would be based, in part, on the removal of soluble carbohydrates, thereby limiting the early fall in pH of the liquor caused by the activities of the aerobes. Too rapid and marked a fall in pH during the very early stages of retting might well retard germination of the spores of the retting bacteria. This hypothesis is in accord with earlier views of Ruschmann (1923) and Allen (1946), but is disputed, in part at least, by Enebo (1947). The latter investigator maintains that the aerobic flora are of no great importance to the appearance of volatile acids in the first phase of retting. This contention is based on determinations of the acetic: butyric acid ratio at different stages of retting. As this ratio was the same at 20 hr. as at the conclusion of Swedish rets, Enebo concluded that the true retting bacteria, which produced butyric acid, were active much earlier than had been believed formerly and that the division of the retting period into "preliminary phase" and "principal phase" by Ruschmann (1923) was purely artificial.

The views of Störmer (1904) and Ruschmann (1923), that the development of aerobic species during the preliminary state of retting, with consequent removal of oxygen from solution, paves the way for the subsequent activities of the anaerobic retting bacteria, have not been disputed by later workers. Indeed, there can be no doubt that the aerobic flora, by lowering the redox potential of the liquor to a level consonant with the germination

of the anaerobic spores, makes possible the comparatively simple industrial application of the anaerobic fermentation process known as retting, without resort to the special equipment and precautions needed to provide an anaerobic environment.

The composition of the aerobic flora of Australian retting liquors has proved to be broadly similar to that found by previous investigators in other countries. Streptococci of the types found here were also reported by Stutzer (1927) and Allen (1946). Paracolon bacteria, which were reported by Enebo (1947) in his study of flax retting in Sweden, were also much more common in Australian retting than the coliform types found by such workers as Störmer (1904), Stutzer (1927), and Allen (1946). The occurrence of large numbers of paracolons in flax retting liquors is of interest since, although this group has received a good deal of attention in relation to gastro-intestinal disorders in recent years, little other work appears to have been carried out with them. In this paper, members of this group have been classified for convenience according to Bergey *et al.* (1948) in the genus *Paracolobactrum* Borman, Stuart, and Wheeler (1944). It is considered, however, that this generic separation is an artificial one and that these organisms are probably variants that have lost, in varying degrees, their activity towards lactose, since this sugar is rarely encountered in their natural environment. Some evidence for this contention is given in a paper by Mushin (1949) who found that some of her cultures regained the ability to ferment lactose rapidly, after repeated subculture in media containing this sugar. A few tests made by the present author have yielded similar results in the adaptation of strains from retting liquor.

Other types of aerobic bacteria isolated from Australian flax retting, namely species of *Flavobacterium*, *Achromobacter*, *Lactobacillus*, and *Microbacterium*, are widely distributed on natural products and their presence is probably adventitious. The isolation of only one species of *Bacillus* confirms the conclusion of several previous investigators that bacteria of this type play no part in anaerobic flax retting. The appearance of large numbers of yeasts in the liquor towards the end of the ret, i.e. when the pH has fallen to between 4 and 5, is in keeping with the usual sequence of events where vegetable material is undergoing microbial decomposition.

It was observed in the present investigation that the lactic streptococci were consistently relatively more numerous in the later stages of retting than the other aerobic bacteria, such as the paracolons and coliforms. This is in accord with the earlier findings of Stutzer (1927) and Allen (1946). Obviously, the streptococci tolerate the acidic conditions prevailing towards the end of a ret (pH 4.2-4.4) better than do the other types. The predominance of streptococci in the later stages of retting probably accounts, in part, for their constituting such a high proportion of the total aerobic organisms isolated here, since approximately equal numbers of colonies were picked from the pour plate cultures of liquor samples taken at each retting stage.

(b) *The Coliform Flora*

Although members of this bacterial group were not recovered in appreciable numbers from the aerobic plate count cultures, the use of more specific methods demonstrated that they are well represented in flax retting liquor. Their numbers and types, however, vary considerably from ret to ret, the range of maximal counts being from 10^2 to 10^8 per ml. With the exception of *Escherichia coli* type II, all of Wilson's (1935) major types and three of the irregular types were found from time to time. It is of interest to note that, even with this isolation method based on acid and gas production in MacConkey's broth, almost 30 per cent. of the cultures recovered were paracolon types.

Of the coliform bacteria isolated from the cultures used for presumptive counts, 11.4 per cent. were *Esch. coli* type I. To this figure may be added the 3.4 per cent. of irregular type II, making a total proportion of 14.8 per cent. of faecal types. This proportion is only slightly lower than that reported by Allen (1946), who found 16.7 per cent. of faecal strains in 150 cultures.

A finding of some considerable interest in the present study was that all but one of the faecal strains was isolated from mill retting liquor. That is to say, while faecal coliform bacteria were present in only one of 18 laboratory rets, they were found in 10 of 11 mill rets, nine of the latter yielding *Esch. coli* type I. The reason for the more frequent occurrence of faecal types in mill retting liquor is not clear at present. However, as thorough cleaning of mill retting tanks between rets is not possible, owing to the rough, pitted nature of the walls and other structural features, it is thought that some form of carry-over from ret to ret may be responsible. Whether this transfer is of bacterial cells, or of some substance favourable to the multiplication of the faecal strains is not evident at this juncture. There is no apparent reason why any one of the various types present should gain the ascendancy by transfer from ret to ret, unless the environment favoured it more than the others but, with such rapidly multiplying bacteria as the coliform group, it seems reasonable to assume that, once introduced, a particular type could persist in a tank and, given a favourable medium, thereby constitute an appreciable proportion of the flora in successive rets. On the other hand, the possibility of transfer of some growth-promoting substance, which is particularly stimulatory to the faecal coliform types, cannot be ruled out. The laboratory retting tanks, which are constructed of stainless steel with a highly polished inner surface, can be much more thoroughly cleaned between rets and the amount of material carried from ret to ret must be very small. Thus, in laboratory rets, it appears unlikely that the flora on the straw itself would be augmented by seeding from the tank walls. Moreover, the presence of faecal coliform strains in only one of 18 laboratory rets studied is in keeping with the fact that these bacteria are not encountered in material free from contamination by animal excreta.

Recalculation shows that, of the coliform bacteria isolated from mill rets, 28.1 per cent. were *Esch. coli* type I and 9.4 per cent. were irregular type II,

a total of 37.5 per cent. of faecal types. Consequently, mill retting effluents may be expected to have a definite effect on the faecal coliform count of any body of water into which they may be discharged. The magnitude of this effect would, of course, vary according to the volume of retting effluent discharged in a given time and the capacity of the lake or flow rate of the river in question, and could only be determined by periodical analyses.

(c) *The Retting Flora*

The method of isolation employed has been shown to have a marked influence on the proportions of retting and non-retting clostridia recovered from heated suspensions of retted flax. Even with the most suitable method found to date, however, namely direct plating on glucose yeast extract agar, approximately equal numbers of both groups are usually recovered. It would now appear that the true retting organisms constitute a relatively small part of the total bacterial flora of retting flax and consequently, it might well be anticipated that their activity would be influenced, to some extent at least, by variations in the composition of the subsidiary flora. Thus, the interrelationships of various non-retting organisms and the retting species may prove a fruitful field for future investigations.

Four distinct types of retting clostridia have been identified in this study of Australian flax retting. Two of these, viz. types I and II, are evidently the agents predominantly responsible for retting in this country, while types III and IV are less frequently present and then only in relatively small numbers, so that their role must be a minor one. Indeed, type IV has only been isolated from three of the large number of flax samples examined. Tests on sterilized flax straw in yeast water, using a number of strains of each type, have revealed noteworthy differences in the respective retting abilities of the four organisms. Type III, *Clostridium felsineum*, proved outstanding with regard to both rate and effectiveness of retting. This observation agrees with earlier findings in this field (Carbone and Tobler 1922; Markova 1940; Ruschmann and Bartram 1943; Enebo 1947). Clostridial types II and IV were less efficient retting agents than *Cl. felsineum*, although apparently effective by qualitative tests in a longer time, while type I, the organism most commonly encountered here, was by far the least active of the four. In fact, many strains of type I left the flax incompletely retted after 7-10 days incubation at 37°C.

The fact that *Cl. felsineum* is not commonly present in large numbers, and that less active and frequently inefficient agents predominate, in Australian flax retting probably bears a significant relationship to the slowness of retting in this country and the frequently under-retted nature of our fibre. This hypothesis and experiments relating thereto were discussed in an earlier publication (Lanigan 1950). However, in the light of the evidence now available, some modification of views expressed then would appear necessary. Initially, it was thought that the active retting agent, *Cl. felsineum*, was sparsely distributed in the soil of our flax-growing districts and, consequently, only appeared in an occasional ret; but Table 6 shows that the direct plating

method led to the detection of this organism in almost half the rets examined in this way. The isolation of this clostridium from such a high proportion of these rets suggests that it may actually be present in most or all of them, but in numbers too small to permit detection, in the absence of a suitable enrichment technique. Thus, it would now appear that the lack of prominence of *Cl. felsineum* in Australian flax retting may be due, not to its infrequent presence on the straw, but rather to environmental factors during retting. There are, of course, many ways in which the environment could prove unsuitable for a particular species and these may be either peculiar to some flax crops or common to all. If the foregoing assumption is correct, then it would be anticipated that inoculation of rets with pure cultures of *Cl. felsineum* would be ineffective, unless substances carried over in the inoculum obviated environmental deficiencies. The author's experiments on rets inoculated with *Cl. felsineum* are incomplete at present and no definite conclusions can yet be drawn from them regarding the value of such inoculation. The following observations are, however, pertinent to this discussion. While the retting of some flax samples is greatly improved by the inoculation, the effect with others is only slight. Moreover, in the relatively few cases investigated to date, it has only been possible to recover *Cl. felsineum* from some of the straw samples at the conclusion of retting. Thus, there is evidence that this clostridium fails to survive, or does not multiply appreciably, following inoculation of some Australian flax rets, and this lends support to the suggestion made above that scarcity of *Cl. felsineum* in our retting may be attributable to environmental factors. As it has been demonstrated that inoculation with this organism leads to marked acceleration and improvement of the retting of some Australian flax straw, it is obviously of great importance that an attempt should be made to establish the underlying causes of its failure to survive and multiply in other rets.

Of the various retting clostridia reported in the literature, only *Cl. felsineum* is accorded specific rank in the current edition of Bergey's Manual (1948). Most of the others are listed as probable varieties of *Cl. butyricum*. There is no doubt, however, that retting clostridia generally, and the plectridial species in particular, exhibit quite different morphological and biochemical characteristics from those of *Cl. butyricum*. It is evident, therefore, that a detailed comparative study of existing cultures of retting bacteria should be undertaken, in order to establish their taxonomic relationships. Three of the four retting agents encountered in the present study differ significantly from currently recognized species of *Clostridium* but it is not the author's intention to name them as new species at this stage, since this would only aggravate an already confused situation. A few comments on clostridial types I, II, and IV may, nevertheless, be justified.

The characteristics of the Australian retting clostridium type I agree well with those of retting organisms described by Weizmann and Hellinger (1940), who considered that their organisms should be classified with Störmer's (1904) plectridial forms as *Clostridium pectinovorum* Störmer, except for the fact that

the Australian strains were micro-aerophilic, as were Störmer's strains (Bergey *et al.* 1948). It is possible, of course, that varieties of *Cl. pectinovorum* differ in the degree of their oxygen tolerance. *Cl. tertium* (Henry) Bergey *et al.* (1948) is the only saprophytic, micro-aerophilic clostridium given specific status by Bergey *et al.* (1948) but, while this organism also forms terminal, elliptical spores, Weizmann and Hellinger's and the author's strains of *Cl. pectinovorum* differ from *Cl. tertium* in liquefying gelatin, actively fermenting potato mash, and in not reducing nitrates. Accordingly, there appear to be good reasons for the recognition of *Clostridium pectinovorum* Störmer as a (micro-aerophilic) species of the genus *Clostridium*.

Type II retting clostridium, an obligate anaerobe, has the general characteristics of the butylic group of clostridia, but does not correspond to any of the species described by Bergey *et al.* (1948). For example, it resembles, in some respects, *Cl. acetobutylicum* but differs significantly from this organism in its morphology, colonial form, fermentation of pectin, and non-fermentation of mannitol and inulin. It appears likely therefore that the Australian retting clostridium type II is a new species.

The yellow-pigmented, retting clostridium type IV exhibits similar biochemical properties to those of *Cl. felsineum*. However, in addition to obvious differences in pigmentation, these two clostridia show marked differences in the morphology of their sporangia and spores and in their colonial form. Apart from two cellulose-digesting clostridia, which are obviously unrelated to clostridium type IV, no yellow-pigmented clostridia are described by Bergey *et al.* (1948). However, de Graaf (1930), mentioned by McClung (1943), described a yellow-pigmented clostridium which he called *Cl. xanthogenum*, and McClung (1943) reported that he had isolated three or four similar types. Unfortunately, descriptions of these organisms are not yet available to the author, so that no comparison of them with local strains is possible. There seems little doubt, however, that at least one species of yellow-pigmented clostridium, other than the cellulose digesters, exists.

(d) *The Non-Retting Anaerobic Flora*

The fact that some 87 per cent. of the non-retting clostridia, isolated in this study of Australian flax retting, proved to be varieties of *Clostridium butyricum* is, perhaps, not unexpected, since such organisms are known to be common in cultivated soils, on plants, and on other natural products. Conditions during retting are obviously favourable for their development, as they are usually present in numbers equalling those of the retting bacteria. It is tempting to speculate whether or not these butyric anaerobes are of assistance to the retting species, for example, by virtue of the known ability of some varieties to fix atmospheric nitrogen. As far as is known, however, this possibility has not been investigated.

All but one of the 151 strains of *Cl. butyricum*-like anaerobes studied by the author failed to correspond in their properties with the varieties listed by Bergey *et al.* (1948). Thus, although they were separable into *butyricum* and

multifermentans types, they consistently fermented mannitol, glycerol, and lactate, thereby showing overlapping properties of these two starch-fermenting varieties. Varieties of *Cl. butyricum* with similar properties to those described here were reported by Tabachnick and Vaughn (1948) to be capable of fermenting tartrates. Thus the biochemical properties of these butyric anaerobics from retted flax, together with the occurrence of five distinct colonial types, emphasize the variability existing in this group of clostridia.

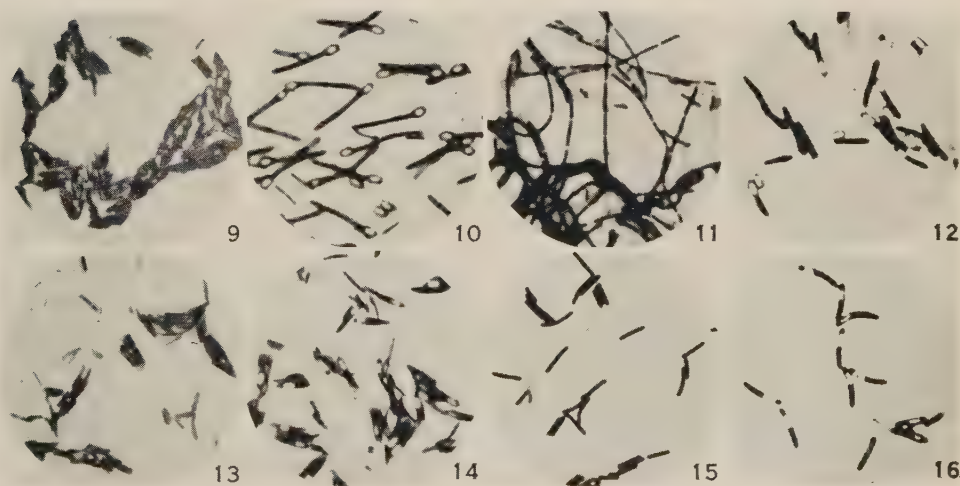
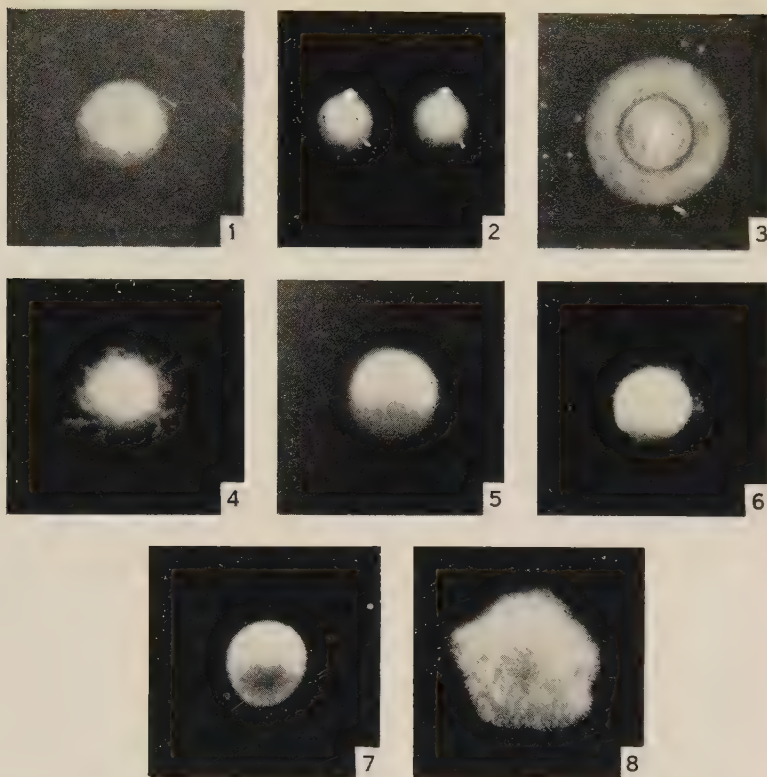
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VI. REFERENCES

- ALLEN, L. A. (1946).—*J. Soc. Chem. Ind.* **65**: 365, 373.
 BEHRENS, J. (1903).—*Zbl. Bakt.* II **10**: 524.
 BEIJERINCK, M. W., and VAN DELDEN, A. (1904).—*Arch. Néerl. Sci.* (2) **9**: 418.
 BERGEY, D. H., *et al.* (1948).—"Manual of Determinative Bacteriology." 6th Ed. (Williams and Wilkins Co.: Baltimore.)
 BORMAN, E. K., STUART, C. A., and WHEELER, K. M. (1944).—*J. Bact.* **48**: 351.
 CARBONE, D., and TOBLER, F. (1922).—*Faserforschung* **2**: 163.
 CARBONE, D., and TOMBOLATO, A. (1917).—*Staz. Sper. Agr. Ital.* **50**: 563.
 D.S.I.R., GREAT BRITAIN (1948).—Flax retting with aeration. Water Pollution Res. Lab. Tech. Pap. No. 10: 10-2.
 ENEBO, L. (1947).—*Acta Agric. Suecana* **2** (3): 319.
 DE GRAAF, J. (1930).—*Ned. Tijdschr. Hyg.* **4**: 219.
 HAYWARD, NANCY J. (1945).—*Proc. Ass. Clin. Path.* **1**: 5.
 HUCKER, G. H., and PEDERSON, C. S. (1930).—New York Agric. Exp. Sta. Tech. Bull. No. 167: 66.
 JENSEN, H. L. (1941).—*Proc. Linn. Soc. N.S.W.* **66**: 276.
 LANIGAN, G. W. (1950).—*Nature* **165**: 516.
 MARKOVA, Z. S. (1940).—*Mikrobiol. (U.S.S.R.)* **9**: 824.
 McCLUNG, L. S. (1943).—*J. Bact.* **46**: 507.
 MCCOY, ELIZABETH, and McCLUNG, L. S. (1935).—*Arch. Mikrobiol.* **6**: 230.
 MUSHIN, ROSE (1949).—*Aust. J. Exp. Biol. Med. Sci.* **27**: 543.
 ORLA-JENSEN, A., and KLUYVER, A. J. (1939).—*Zbl. Bakt.* II **101**: 257.
 ROSSI, G. (1904).—*Annu. Scu. Agr. Portici* **5**.
 ROSSI, G., and CARBONE, D. (1909).—*Annu. Scu. Agr. Portici* **9**.
 RUSCHMANN, G. (1923).—*Grundlagen der Röste*, Leipzig.
 RUSCHMANN, G., and BARTRAM, H. (1943).—*Zbl. Bakt.* II **105**: 326, 433.
 RUSCHMANN, G., and BAVENDAM, W. (1925).—*Zbl. Bakt.* II **64**: 340.
 STÖRMER, K. (1904).—*Zbl. Bakt.* II **13**: 171, 306.
 STUTZER, M. J. (1927).—*Zbl. Bakt.* II **69**: 164.
 TABACHNICK, J., and VAUGHN, R. H. (1948).—*J. Bact.* **56**: 435.
 WEIZMANN, C. H., and HELLINGER, ESTHER (1940).—*J. Bact.* **40**: 665.
 WILSON, G. S., *et al.* (1935).—The bacteriological grading of milk. Med. Res. Coun. Spec. Rep. No. 206. (H.M.S.O.: London)
 WRIGHT, B. M. (1943).—Army Pathology Service, Great Britain. Current Notes, No. 9.

BACTERIAL FLORA OF AUSTRALIAN FLAX RETTING



EXPLANATION OF PLATE 1

Figs. 1-8.—The colonies illustrated were grown on glucose yeast extract agar, at 37°C. x10.

Figs. 1-3.—Retting clostridium type I.

Fig. 1.—Low convex, butyrous colony; 3 days.

Fig. 2.—Pulvinate, rubbery colony; 2 days.

Fig. 3.—Collapsed form of colony in Figure 2. The inner ring is the original outline; 3 days.

Figs. 4-6.—Retting clostridium type II.

Fig. 4.—Typical colony with myceloid margin; 3 days.

Fig. 5.—Smooth, regular, viscid colony; 3 days.

Fig. 6.—Colony type intermediate between those in Figures 4 and 5; 3 days.

Figs. 7-8.—Retting clostridium type IV.

Fig. 7.—Typical colony; 3 days.

Fig. 8.—Larger, irregular colony on sparsely seeded plate; 3 days.

Figs. 9-16.—Photomicrographs are of Gram-stained preparations from cultures on glucose yeast extract agar, at 37°C. x900.

Fig. 9.—Vegetative cells of retting clostridium type I.

Fig. 10.—Sporangia and spores of retting clostridium type I.

Fig. 11.—Vegetative cells of retting clostridium type II.

Fig. 12.—Sporangia and spores of retting clostridium type II.

Fig. 13.—Vegetative cells of *Cl. felsineum*.

Fig. 14.—Sporangia and spores of *Cl. felsineum*.

Fig. 15.—Vegetative cells of retting clostridium type IV.

Fig. 16.—Sporangia of retting clostridium type IV.

VARIATION IN THE ALKALOIDS OF CLONES OF NORTHERN *DUBOISIA MYOPOROIDES* R. BR.

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Summary

Samples of leaf were obtained from seven clones of the northern or hyoscyne type of *Duboisia myoporoides* for three successive summers at Canberra, Australian Capital Territory, and from the same clones for a winter and a summer at Nambour, south-eastern Queensland. The alkaloids were separated by partition chromatography and the hyoscyne determined quantitatively. Some of the other alkaloids present were identified.

Consistent differences in hyoscyne content were evident between certain of the clones and it is presumed that they differ genetically in their capacity to produce this alkaloid. The average percentage of hyoscyne per clone ranged from 0.6 to 1.5 per cent. of the dry weight of the leaf.

The yield of both total alkaloids and of hyoscyne were of the same order of magnitude at Canberra and Nambour but there was considerable variation between samplings at each location. Interaction was evident between the yield of hyoscyne from individual clones and the year or season of sampling.

Alkaloids other than hyoscyne accounted for from 30 to 90 per cent. of the alkaloids found in individual samples. They comprised in various combinations hyoscyamine, valeroidine, an unidentified alkaloid with a high melting point picrate, and one or more unidentified alkaloidal substances.

I. INTRODUCTION

Preliminary investigations (Loftus Hills, Trautner, and Rodwell 1945) have shown that both heredity and environment are important in determining the proportion of hyoscyne in leaves of *Duboisia* species. Certain major geographical races have been described (Loftus Hills and Kelenyi 1947) but an evaluation of the variation within these groups was not possible until suitable quantitative analytical methods were developed. The successful application of the partition chromatography of Evans and Partridge (1948) to *Duboisia* leaf extracts, and the use of a modification of the method of Trautner, Neufeld, and Rodwell (1948) for the quantitative estimation of hyoscyne have enabled further progress to be made.

This paper describes the variation in total alkaloids and in the hyoscyne content of seven clones† from the northern or hyoscyne-dominant group of *Duboisia myoporoides* for three successive summers at Canberra, A.C.T., and a winter and a summer at Nambour, south-eastern Queensland (see representative meteorological data in Table 1). An exhaustive investigation of the

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† Four of the leaf samples of the seventh clone were too small for assay, and were replaced by composite samples from siblings of the original selection.

genotypes available within any particular zone was clearly impracticable. The project was therefore confined to a study of the environmental interaction of a limited number of clones, selected at random in regard to alkaloid propensities, but with some regard to geographical origin and to the more obvious variations in vegetative form observed in a number of seedling progenies being grown for selection. The authors are unaware of any comparable investigations with other alkaloid-bearing species.

TABLE 1
METEOROLOGICAL DATA FOR CANBERRA AND NAMBOUR

	Normal Mean Minimum Temperature (°F.)		Normal Mean Maximum Temperature (°F.)		Normal Mean Relative Humidity (%)		Mean Annual Rainfall (in.)	Latitude	
	July	Jan.	July	Jan.	July	Jan.			
Canberra	33	55	52	82	83	53	23	35°	20'S.
Nambour (Gympie)	42	67	71	88	75	74	47	26°	11'S.

II. MATERIALS AND METHODS

(a) Botanical

From 10 to 30 vegetative propagants were struck from each of a number of individuals selected from seedling progenies of *D. myoporoides* growing at Canberra, A.C.T. One-half of each of these clonal populations were transplanted into the field at Canberra and the remainder were established similarly at Nambour. Seven of them were selected for further study on the basis of maximum vigour in both environments, diversity of geographical origin of the parental seed, and diversity of leaf shape and plant form.

The location of the parent trees from which the selected seedlings were derived, together with the northern and southern limits of distribution of the hyoscine-dominant type of *D. myoporoides* are shown in Figure 1. Clones 437-2, 455-1, 468-1, and to a lesser extent 355-1 and 428-1, may be regarded as vegetatively normal for the group, whereas 327-1, with small, narrow, olive-green leaves, and 370-1 with glossy, prominently veined, dark green leaves, are atypical. The vegetative characters vary throughout the region of occurrence but not apparently in any regular manner.

Fertility was maintained during the course of the experiment by regular dressings of NPK fertilizer. After the initial watering in of the transplants growth was maintained by natural rainfall. Leaf samples were taken during the first, second, and third summers after establishment in the field at Canberra, and in July and November 1947 at Nambour. The methods used for sampling and drying were the same as those described previously (Loftus Hills, Trautner, and Rodwell 1945).



Fig. 1.—The geographical origin of the seed from which the clones of northern *D. myoporoides* were established.

(b) Chemical

The method of Loftus Hills, Trautner, and Rodwell (1945) was designed to give a rapid overall picture of a large number of small samples. It gave an approximate (possibly too high) figure for total alkaloids, and a general assessment of the nature of the main alkaloids present, based on the identification of the picrates. The methods used in the present investigation gave a more exact figure for the total alkaloids, a quantitative evaluation of the hyoscyne, and allowed of the identification of some of the other alkaloids.

The total alkaloids obtained by the earlier method were estimated by titration of an organic extract without passage through water or other purification. The methods used in the present investigation entailed repeated cycles through water and organic solvent, until a constant titration figure was reached. This procedure led, in every case, to a substantial decrease in the titration of bases. The decrease was greatest between the first and second titrations and became less with each succeeding cycle. In some cases the second and third titrations were sufficiently close for the third one to be taken as final but more often four or five cycles were necessary. The nature of the titratable material discarded by this procedure is not known—a few chromatograms of the first (i.e. least pure) ether extracts gave no indication of the presence of additional bases in any significant amount. The final titration of bases was occasionally as little as 30 per cent. of the first one, but was usually about 70 per cent. The figures were similar whether the chloroform extract was titrated with *p*-toluenesulphonic acid, or the aqueous extract titrated with sulphuric acid.

Two main methods were used. The first was based on the estimation of hyoscyne as the picrate, and the second involved the separation of the bases by partition chromatography and the subsequent estimation of hyoscyne by titration.

(i) *The Picrate Method*.—Ten g. of dry, powdered leaf was extracted by shaking with successive 150 ml. portions of cold, purified methanol until a small portion, on evaporation with dilute acid, showed no reaction with Mayer's reagent (K_2HgI_4). The methanol was evaporated under reduced pressure and the residue acidified to congo red with 1 per cent. sulphuric acid. The precipitate was broken up, stirred thoroughly, filtered under reduced pressure, and the filter cake washed with very dilute acid until free from alkaloids.

The filtrate was transferred to a separating funnel, washed once with chloroform which was extracted with dilute acid, and then discarded; the acid wash being added to the main extract. The main extract was made alkaline to phenolphthalein with solid sodium carbonate and exhausted with chloroform. The chloroform was run off carefully through a dry filter paper, concentrated to half its volume and titrated with N/20 *p*-toluenesulphonic acid as in the method described by Loftus Hills, Trautner, and Rodwell (1945), to give the first titration of bases.

After titration the chloroform extract was exhausted with dilute acid, the acid extract made alkaline with sodium carbonate, and exhausted with chloroform. The chloroform was run off through a dry filter paper, concentrated, and then titrated with N/20 *p*-toluenesulphonic acid to give the second titration of bases.

This purification cycle was repeated until two successive titrations did not differ by more than 0.75 ml. N/20 acid, equivalent to 0.1 per cent. of bases calculated on a molecular weight of 300.

The final chloroform solution was exhausted with dilute acid and, after being made alkaline, shaken back into chloroform. The chloroform solution

was dried and titrated with standard N/20 picric acid in chloroform with dimethylaminoazobenzene as indicator. One-third volume of benzene was added, the solution left standing for 24 hr., and then the precipitated hyoscyne picrate was filtered off, dried, and weighed. The mother liquors, after removal of hyoscyne picrate, were examined by the addition of further small quantities of benzene and light petroleum and by the removal of all organic solvents and recrystallization of the oily residue from water, in an attempt to separate the other alkaloid picrates present.

(ii) *The Chromatographic Method.*—A continuous extraction cell of stainless steel was designed to facilitate several of the laborious operations involved in this method (Fig. 2). The most suitable dimensions depend on the characteristics of the shaker to be used but the sizes shown proved satisfactory with a shaker consisting of a box swinging on a 3 in. arc of a circle of 12 in. radius at 50 cycles per min.

Five g. of ground leaf was introduced into a compartment of the cell and carefully moistened with 40 ml. of 10 per cent. sodium carbonate. Standard N/10 sulphuric acid (15 ml.) was introduced into the other compartment and ether added until the partition was well covered. The cell was set lengthwise in the shaking machine for 24 hr., during which time the ether became green or yellow-green, but the acid remained colourless. The acid was pipetted out, the compartment rinsed twice with water, and the excess acid titrated with standard N/10 sodium carbonate, using methyl orange as indicator. A further 2 ml. of standard acid was introduced, shaken for several hours, pipetted out and the excess titrated as before. Extraction was continued until no more than 0.5 ml. of N/10 acid was used in a 12-hr. run. Complete extraction from the leaf was slow, often taking 6-7 days, but the time required for manipulation was short and the extraction proceeded steadily without the use of heat. No evidence of hydrolysis was observed.

The titrated solution was made alkaline by the addition of solid sodium carbonate and replaced in the cell. Acid was placed in the other half as before and the second stage completed in a similar manner. This process was repeated until the total titration values at the completion of successive cycles did not differ by more than 0.4 ml. of N/10 acid, equivalent to 0.1 per cent. of bases calculated on a molecular weight of 300. The first cycle was usually complete in 48 hr., and later ones within 24 hr.

The final aqueous solution was made alkaline and exhausted, either by repeated extraction with chloroform in a separating funnel or in a continuous liquid-liquid extractor. In the latter case the chloroform slowly darkened to orange as extraction proceeded. In each case the resulting chloroform solution was evaporated to 10 ml., and an aliquot of the solution equivalent to 1 g. of the original plant material was titrated against N/100 *p*-toluenesulphonic acid in chloroform, with dimethylaminoazobenzene as indicator. In spite of several lengthy extractions, a further loss of bases occurred during this transfer. The residual aqueous extract was negative to Mayer's reagent and no further basic material could be extracted with chloroform. Complete transfer of known

mixtures of hyoscyne and hyoscyamine was effected under similar conditions and without difficulty. It is assumed that this final titration of bases in chloroform gave the best estimation of the total alkaloids present.

The alkaloids were separated on a chromatographic column of the type described by Evans and Partridge (1948), and the various bases estimated by titration. The support used for the stationary phase was "Hyflo-Supercel,"

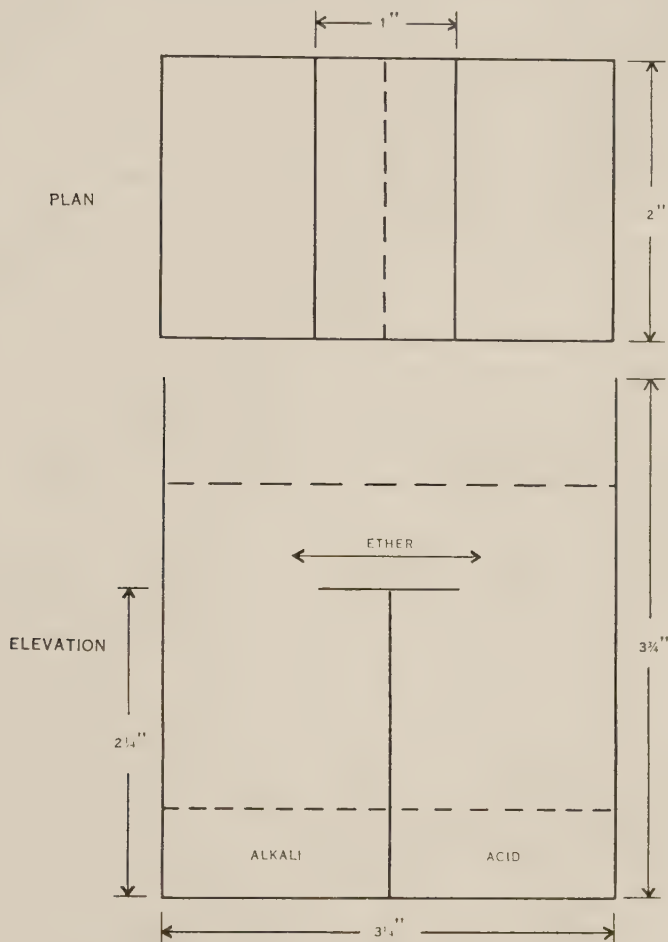


Fig. 2.—The cell for continuous extraction of the alkaloids.

a standardized kieselguhr prepared by the John's-Manville Co., U.S.A. Twenty g. of the material were moistened evenly with 4 ml. of M/2 phosphate buffer at pH 7.0, and packed into a Pyrex glass tube 40 cm. long by 1 cm. in diameter, with a stopcock sealed on the lower end. Packing was done carefully by first partly filling the tube with ether saturated with the buffer and then introducing the supercel in small lots, packing each lot firmly with a perforated disc

plunger. After the packing was completed 100 ml. of buffered ether were run through under gravity. The column was then mounted on a mechanically operated fraction collector arranged so that 5 ml. aliquots were delivered to individual test tubes set on the periphery of an intermittently rotating circular platform.

An aliquot of the chloroform extract equivalent to 1 or 2 g. of dried leaf was used for the separation of the alkaloids. After evaporation of the chloroform the residue was taken up in ether, introduced into the top of the column, and allowed to soak in. A reservoir containing ether that had previously been saturated with the buffer was attached to the top of the column and a constant air pressure of 15-20 mm. of mercury applied.

The ether was evaporated from each 5 ml. aliquot and the residue dissolved in chloroform and titrated with N/100 *p*-toluenesulphonic acid. Development with ether was continued for 60 aliquots (300 ml.), or more if titratable bases were still appearing in the eluate. Development was then continued with chloroform saturated with the buffer until no further bases appeared. If the total titration value of the eluate was still appreciably lower than that determined just before chromatography, development was continued with 1:1 butanol-chloroform.

Hyoscine first appeared at about the fifth aliquot and reached its maximum concentration very rapidly. It was usually recovered *in toto* by the fifteenth aliquot although, particularly with plant extracts, the titration did not always immediately fall to zero.

Hyoscyamine generally appeared at about the thirtieth aliquot, rising and falling very slowly, so that often it was not completely recovered until the sixtieth aliquot. However, its exact position varied from time to time, even when dealing with replicate samples from the same solution of pure alkaloids.

Three of the minor alkaloids of *Duboisia* described by Barger, Martin, and Mitchell (1937) were also investigated. Tigloidine appeared in the eluate fractions together with hyoscine but it has not yet been possible to devise a means of separating them. Valeroidine was eluted with ether and appeared between hyoscine and hyoscyamine, although some variation in its position has been observed, and overlapping, particularly with the latter, may occur. Base Z, which is a mixture of poroidine and isoporoidine only appeared after development with chloroform.

In certain cases the alkaloids appearing in the region of particular peaks were identified by examination of their picrates. The titrated solutions were exhausted with dilute acid, the acid extract made alkaline with ammonia, and the resulting solution exhausted with ether. The ether was then layered over saturated aqueous picric acid in a test tube, and allowed to stand while crystallization proceeded at the interface. Hyoscine was particularly easy to identify by this means, and the melting point of the crystals was seldom more than a few degrees below that of the pure salt. The peak representing a mixture of hyoscine and tigloidine yielded a picrate with a melting point close to that of hyoscine picrate, so that tigloidine could not be detected by the method.

If hyoscyamine was present in reasonable quantity it could usually be identified, although overlapping impurities such as valeroidine and other minor alkaloids were apparently often also present in the region in which hyoscyamine occurs. This general problem, together with the characterization of other peaks, is still under investigation.

The recovery of total alkaloids from the column ranged from 100 to 65 per cent. Results with known mixtures showed that when the recovery was less than 100 per cent. a higher proportion of hyoscine was recovered than of alkaloids of greater basicity. Duplicate aliquots of each extract were fractionated separately and the amount of hyoscine calculated from the duplicate showing the greater recovery.

In the first of the five groups of samples assayed by this method (Canberra 1946-1947) the ground leaf was extracted with methanol in the manner described for the picrate assay, instead of with ether in the extraction cell. The former procedure generally led to somewhat higher titrations for total bases than did the latter.

TABLE 2
TOTAL ALKALOID CONTENT OF CLONES OF NORTHERN *D. MYOPOROIDES* AT
DIFFERENT TIMES AND PLACES

Clone No.	Total Alkaloids* as Percentage of Dry Matter of Leaf					Mean† per Clone
	Canberra, 1946-47 Summer	Canberra, 1947-48 Summer	Canberra, 1948-49 Summer	Nambour, 1947 Winter	Nambour, 1947-48 Summer	
327-1	2.0	5.0	3.3	3.3	4.3	3.6
355-1	2.0	1.2	2.0	3.7	2.5	2.7
370-1	2.0	3.0	1.5	1.1	2.7	2.1
428-1	1.7	3.3	1.5	2.6	2.7	2.4
437-2	2.0	2.7	3.0	2.1	3.3	2.6
455-1	1.6	3.6	2.6	2.3	3.8	2.8
468-1	1.3	3.6	2.5	3.3	2.6	2.9
Mean ‡ per sampling	1.8	3.2	2.3	2.6	3.1	

* Calculated from the final titration of bases immediately prior to the separation of the alkaloids in the chromatographic method.

† Minimum difference for significance at the 5% level = 0.92.

‡ Minimum difference for significance at the 5% level = 0.79.

(c) Results and Discussion

(i) *Total Alkaloids*.—The percentages of total alkaloids calculated from the final titration of bases in chloroform are given in Table 2. The individual values range from 1.1 to 5.0 per cent. and the means per clone from 2.1 to 3.6 per cent. The differences between clones are of marginal significance ($P = 0.07$). There is no significant difference between locations but the differences between times within locations are highly significant ($P < 0.01$).

Differences between clones and between times of harvest are not unexpected but it is of interest that a subtropical coastal species may produce as great a quantity of alkaloids when grown in an inland environment having a continental climate as when cultivated in its original habitat.

(ii) *Hyoscyne*.—The hyoscyne content of all five sets of samples was determined by the chromatographic method, and also, in three of them, by the picrate method. The percentages of hyoscyne found by both methods are shown in Table 3. The results are in reasonably good agreement, with the exception of clone 437-2, which often failed to yield picrate precipitates, but gave a positive identifiable hyoscyne component when fractionated in the column.

TABLE 3
HYOSCINE CONTENT OF CLONES OF NORTHERN *D. MYOPOROIDES* AT DIFFERENT TIMES AND PLACES

Clone No.	Hyoscyne as Percentage of Dry Matter of Leaf								Mean† per Clone
	Canberra, 1946-47 Summer	Canberra, 1947-48 Summer	Canberra, 1948-49 Summer	Nambour, 1947 Winter	Nambour, 1947-48 Summer				
	Chrom. Method	Picrate Method	Chrom. Method	Picrate Method	Chrom. Method	Picrate Method	Chrom. Method		
	Method	Method	Method	Method	Method	Method	Method		
327-1	0.9	1.5	1.6	1.5	1.5	0.6	0.7	2.6	1.5
255-1	0.9	—	1.0	—	—	—	1.7	1.1*	1.2
370-1	0.3	1.0	1.3	0.8	0.6	0.6	0.4	1.7	0.9
428-1	0.5	0.8	1.1	0.8	0.5	0.6	0.6	1.5	0.8
437-2	0.2	0.0	0.3	0.2	0.3	0.0	0.3	1.7	0.6
455-1	0.5	2.0	1.9	1.9	1.8	0.6	0.5	2.1	1.4
468-1	0.5*	1.7*	1.8*	1.7*	1.6*	0.9	1.2	1.1*	1.2
Mean per sampling† excluding clone 355-1	0.48		1.33		1.05		0.62	1.78	

* A composite sample from a number of siblings of the clone.

† Minimum difference for significance at the 5% level = 0.50.

‡ Minimum difference for significance at the 5% level = 0.46.

Although there is considerable variation in the relative yields of the clones due to environmental factors and possibly also to errors of assay, it is clear that some clones produce hyoscyne in greater quantity on the average than others; as the overall differences between them are statistically significant ($P < 0.01$). The interaction between clones and seasons within places is greater than that between clones and places, which suggests that clones may rank similarly in different environments. However, only two locations are involved in the present data and it is possible that interactions may occur with other geographical locations.

During the course of preliminary experiments, over 200 samples were obtained at various times from many seedling lines and clones of *D. myoporoides*

growing in a number of plots. These were all assayed by a semi-quantitative method based on the characterization of the alkaloid picrates (Loftus Hills, Trautner, and Rodwell 1945), the amounts of hyoscyne picrate being rated visually on a scale giving an observed total maximum score of 8. On this basis 15 samples of 437-2 and related seedlings gave an average rating of 0.5 and 20 samples of family 327 an average of 4.0.

It is clear that a range of genotypes exists within the northern section of *D. myoporoides*. Clone 437-2 is typical of those yielding appreciable quantities of hyoscyne only under exceptional circumstances, and 327-1 of those yielding substantial amounts of the alkaloid under most circumstances. A selection programme, particularly in relation to a specific geographical environment, should be effective in establishing high hyoscyne-yielding clones.

The geographical origin of the seed from which the clones were derived does not appear to have influenced the hyoscyne content in any regular manner, the maximum and minimum values being observed in clones having their origin about the middle of the zone of distribution, and intermediate values being recorded from both the northern and southern geographical extremities. It should be noted that we have been dealing only with a few individual seedling isolates and it is impossible to generalize regarding regional population trends from the results presented. It does not follow, for example, that trees grown from bulk seed collected at Grafton (437-2) will prove inferior in hyoscyne content to material from the Coolangatta area (455-1, 468-1).

Visual estimates indicated that the yield of leaf per tree was of the same order for each of the seven clones. For example there was little if any difference in the weight of leaf produced by clones 437-2 and 455-1, and hence the considerable differences between them in percentage of hyoscyne may be regarded as indicative of differences in yield of hyoscyne per tree and per unit area.

The differences in average hyoscyne content between samplings are statistically highly significant ($P < 0.01$); the concentration of hyoscyne being least for the first sampling after establishment at both centres and greatest for the summer harvest at Nambour. It is noteworthy that the hyoscyne content of the clones was not substantially different in the two contrasting environments.

Hyoscyne seldom comprised more than two-thirds of the total alkaloids found and occasionally represented as little as one-tenth of the whole. In Table 4 the hyoscyne figures are given as percentages of the total alkaloids. There was considerable variation, and little can be deduced beyond the fact that the alkaloids of clone 437-2 were frequently characterized by an unusually low proportion of hyoscyne.

The relationship of the amount of hyoscyne to the other alkaloids present is of interest, as it may give some indication whether production is limited by competition for common precursor materials or whether hyoscyne synthesis proceeds independently of that of other alkaloids.

No correlation was found within clones at different times between percentage of hyoscyne and percentage of alkaloids not hyoscyne ($r = -0.0094$).

This result is in conformity with an hypothesis of independent production but other interpretations are possible* and the final solution must await further experimental evidence.

TABLE 4
PROPORTION OF HYOSCINE IN ALKALOIDS FROM CLONES OF NORTHERN *D. MYOPOROIDES*
AT DIFFERENT TIMES AND PLACES

Clone No.	Hyoscyne Content as Percentage of Total Alkaloids Found					Mean per Clone
	Canberra, 1946-47 Summer	Canberra, 1947-48 Summer	Canberra, 1948-49 Summer	Nambour, 1947 Winter	Nambour, 1947-48 Summer	
327-1	43	42	47	27	60	44.5
355-1	61	93	—	65	42	65.3
370-1	16	63	37	34	59	41.6
428-1	28	38	38	30	57	38.1
437-2	11	13	13	15	56	21.7
455-1	36	68	78	20	59	51.2
468-1	39*	61*	72*	46	42*	51.2
Mean per sampling	33.4	54.0	47.5	33.9	53.6	

* A composite sample from a number of siblings of the clone.

(iii) *Alkaloids other than Hyoscyne*.—Alkaloids other than hyoscyne accounted for from about 30 to 90 per cent. of the total alkaloids found. Even in samples yielding maximum amounts of hyoscyne, nearly half the alkaloids remain unaccounted for. Efforts are now being made to characterize these substances so only preliminary data are presented. Some information has been obtained from the chromatograms, some by the examination of picrates prepared from the filtrates after the removal of hyoscyne picrate, and from bulk extracts from four of the clones at Canberra in 1948. Positive identification of one alkaloid does not exclude the possible presence of additional alkaloids in major amounts, and in fact in certain cases where hyoscyamine has been identified other alkaloids are indicated by the presence of further major peaks in the chromatograms. Similarly, failure to establish the presence of identifiable alkaloids does not necessarily exclude their presence, although in such cases they are unlikely to occur in major amounts.

Typical chromatograms are shown in Figure 3. Figure 3A is typical of samples high in hyoscyne and relatively free from other alkaloidal substances. Two-thirds of the alkaloids recovered from this column are accounted for by the clearly defined hyoscyne peak. The remaining third is made up by two unidentified peaks, one lying between hyoscyne and the expected position for hyoscyamine, and the other in the chloroform zone. Figure 3B represents a

* An alternative explanation is that the ratio of hyoscyne to other alkaloids changes as the total amount of alkaloids changes, the change in the ratio with total amount to be such as to give no correlation in the absolute amounts of each produced. Such an hypothesis cannot be excluded even though the correlation within clones between the ratio of hyoscyne to remaining alkaloids and the total amounts is not significant ($r = +0.1049$).

sample yielding appreciable amounts of both hyoscyne and hyoscyamine. In contrast to the previous chromatogram the successive titration values rarely fall to the base level and it would appear that at least four substances of different basicities are present. A still greater contrast is provided by the sample of 437-2 illustrated in Fig. 3C. In this case only a small amount of hyoscyne appeared, the major alkaloids being hyoscyamine (m.p. of picrate = 167°C.) and an unknown represented by a prominent peak in the chloroform zone (m.p. of picrate = 206°C.).

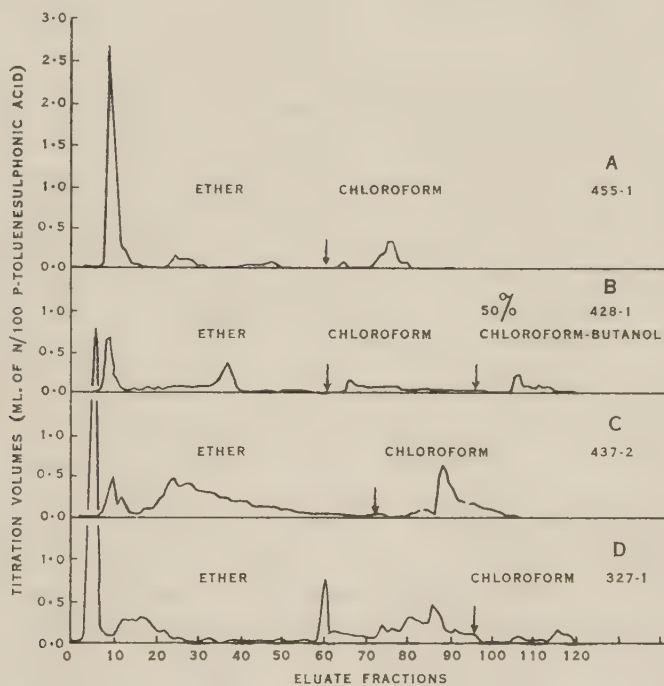


Fig. 3.—Typical chromatograms showing the bases eluted through a buffered kieselguhr column by successive 5 ml. aliquots of solvent, for four contrasting leaf samples from clones of northern *D. myoporoides*.

An approximate estimate of the amount of hyoscyamine was made from some of the chromatograms where the alkaloid appeared as a well-isolated peak and could be identified. The values ranged from 0.3 to 1.2 per cent. It only appeared in quantity at the first sampling after transplanting at both locations when it was positively identified in nine of the 14 samples. Seasonal conditions at the two places are unrelated and it seems possible that the effect may be associated with establishment factors rather than environmental conditions in general. The data do not establish that the presence or absence of hyoscyamine is an inherent character of any particular clone, although unpublished data concerning further material of northern *D. myoporoides* suggests that the tendency to produce hyoscyamine is in fact a genetic variable within the group.

Valeroidine was found in the bulk extract of clone 327-1 to the extent of 0.2 per cent. of the dry weight of the original leaf, but was not found in the other three clones extracted in bulk. The chromatogram is shown in Figure 3D. A substantial peak is apparent in the expected position for valeroidine. Although valeroidine could not be positively identified in the other four samples of 327-1, characteristic valeroidine peaks were apparent in three of them. Occasional samples in certain of the other clones showed minor peaks in the expected position but they could not be positively identified. It does appear that clone 327-1 has a greater capacity for the production of valeroidine than the other six, and in fact it may be that some of them are incapable of synthesizing that alkaloid.

Barger, Martin, and Mitchell (1937) found valeroidine in small amounts in leaf of *D. myoporoides* of unstated origin. Its occurrence in larger quantities in a particular clone suggests the interesting possibility that some at least of the so-called minor alkaloids of *Duboisia* may occur in major amount in certain genotypes of infrequent occurrence. The character of such individual trees would be lost in bulk collections. Support to the idea is given by the earlier observation (Loftus Hills, Trautner, and Rodwell 1945) that certain individual trees yield an as yet unidentified alkaloid with a high melting point picrate in major amounts.

Of the 10 samples for which additional information regarding the alkaloids other than hyoscyne was obtained by examination of the filtrates after the removal of hyoscyne picrate, six yielded appreciable quantities of picrate melting between 200° and 220°C. The data are compatible with the suggestion that clone 428-1 yields this unknown alkaloid in greater quantity than do some other clones, such as 370-1.

In general therefore it seems that the alkaloids other than hyoscyne include singly or in various combinations hyoscyamine, valeroidine, an unknown alkaloid with a picrate of high melting point, and one or more other alkaloids of unknown character and composition. The pattern of these alkaloids remains indefinite and it has not yet been possible to relate their appearance to either season or place.

III. CONCLUSIONS

(1) Consistent differences in hyoscyne content occur among clones derived from seed originating within the zone of distribution of the northern or hyoscyne type of *Duboisia myoporoides*. The most probable explanation is that the clones differ genetically in their capacity to produce hyoscyne. The variation does not appear to be related in any regular manner to the geographical origin of the seed from which the clones were derived, or to their vegetative characters.

(2) The average percentage of hyoscyne in seven clones sampled for three summers at Canberra, A.C.T., and a summer and winter at Nambour, Queensland, ranged from 0.6 to 1.5 per cent. with values for individual samples ranging from 0.2 to 2.6 per cent.

(3) There was considerable variation between samplings in each of the two locations, but the yield of hyoscyne was of the same order of magnitude

at both. Some interaction occurred between location and time of sampling, and the yield of hyoscyne from individual clones.

(4) Alkaloids other than hyoscyne comprised from 30 to 90 per cent. of the alkaloids found in individual samples. The amount of hyoscyne present was not related to the amount of other alkaloids. The other alkaloids included singly or in various combinations hyoscyamine, valeroidine, an unidentified alkaloid having a picrate of high melting point, and one or more additional unidentified alkaloids.

(5) The presence of hyoscyamine in quantity was characteristic of the first sampling in both environments, but genetic differences in the tendency to produce hyoscyamine were not established.

(6) Valeroidine appeared in quantity in one clone. The tendency to produce it is probably influenced by both environmental and genetic factors.

(7) An unidentified alkaloid with a high melting point picrate appeared frequently. The tendency to produce it is probably influenced by both environmental and genetic factors.

(8) The total alkaloid content varied from year to year at Canberra, and from winter to summer at Nambour, but was of the same order of magnitude at both places. There was some indication that the total alkaloids were influenced by the genotype, but the data were not conclusive.

IV. ACKNOWLEDGMENTS

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V. REFERENCES

- BARGER, G., MARTIN, W. F., and MITCHELL, W. (1937).—The minor alkaloids of *Duboisia myoporoides*. I. *J. Chem. Soc.* **1937**: 1820-23.
- EVANS, W. C., and PARTRIDGE, M. W. (1948).—The partition chromatography of alkaloids. I. Solanaceous alkaloids. *Quart. J. Pharm.* **21**: 126-37.
- LOFTUS HILLS, K., and KELENYI, G. P. (1946).—A preliminary report upon the cultivation of *Duboisia* spp. *J. Coun. Sci. Industr. Res. Aust.* **19**: 359-75.
- LOFTUS HILLS, K., TRAUTNER, E. M., and RODWELL, CYNTHIA N. (1945).—A preliminary report upon variation in the nature and quantity of the main alkaloids in *Duboisia myoporoides* and *Duboisia leichhardtii*. *J. Coun. Sci. Industr. Res. Aust.* **18**: 234-53.
- TRAUTNER, E. M., NEUFELD, O. E., and RODWELL, CYNTHIA N. (1948).—The titration of hyoscyne, hyoscyamine and atropine with picric acid in chloroform. Separation and fractionation of the picrates. *Aust. Chem. Inst. J. and Proc.* **15**: 55-61.

THE NATURE OF CARBONIC ANHYDRASE FROM PLANT SOURCES

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Summary

Carbonic anhydrase activity in leaves of plants is low compared with that in animal tissues.

In the absence of cysteine the activity of crude leaf extracts was readily lost.

A method of purification of the enzyme from crude leaf extracts is described. The most active preparation had a catalytic activity about 50-60 times that of the crude leaf extract.

During purification the enzyme was inactivated by dialysis in the absence of cysteine, by lead acetate, acetone, and by high concentrations of ammonium sulphate. The partially purified product contained 0.056 per cent. zinc, not removable by dialysis.

The enzyme is heat-labile.

The relation of activity to pH is described; optimum activity occurs within the range pH 6-8.

Activity of the enzyme preparation is inhibited only by relatively high concentrations of cyanide, sulphocyanide, azide, and sodium sulphide. Sulphanilamide in relatively high concentrations does not inhibit activity. Activity of the preparation is strongly inhibited by *p*-chlormercuribenzoate and by sodium arsenite; their inhibitory effects are reversible, reactivation occurring by the addition of simple thiol compounds such as cysteine and reduced glutathione.

Polarographic investigation suggests the presence of sulphydryl groups in the enzyme preparation.

The enzyme preparation from plant sources differs from that from animal sources in its inactivation during dialysis in the absence of cysteine, its inactivation by lead acetate, acetone, and ammonium sulphate, its relative insensitivity to inhibitors of metal proteins and to sulphanilamide, and in its strong and reversible inactivation by sulphydryl inhibitors.

It is suggested that carbonic anhydrase from plant sources is a different enzyme from that occurring in animal tissues.

I. INTRODUCTION

References to carbonic anhydrase activity in plants are few. Neish (1939) reported carbonic anhydrase activity in species of *Trifolium*, *Onoclea*, and *Arctium*; Day and Franklin (1946) found activity in leaves of some but not all species of *Sambucus* studied by them; Bradfield (1947) found activity in leaves of several species and investigated the effects of some inhibitors on the crude leaf extract.

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In this paper partial purification of the enzyme from leaves of spinach beet (*Beta vulgaris*) has been attempted and some properties of the product described.

II. CARBONIC ANHYDRASE ACTIVITY IN CRUDE LEAF EXTRACTS

(a) Measurement of Activity

For measurement of activity a manometric method using the boat apparatus of Meldrum and Roughton (1934) was used; this depends upon the rate of evolution of CO_2 from sodium bicarbonate solution mixed with phosphate buffer at pH 6.8. In the arms of the flask were placed 2 ml. of 0.2M NaHCO_3 solution dissolved in 0.02M NaOH and 2 ml. of 0.2M mixed phosphate buffer to which was added 0.5 ml. of the plant extract. All estimations were carried out at 5°C .

The unit of activity adopted was the "enzyme unit" (E.U.) of Meldrum and Roughton, but measured at 5°C . instead of 15°C ., and defined as the amount of enzyme preparation which, when dissolved in 4 ml. of phosphate-bicarbonate mixture, gives a value of $(R - R_0)/R_0$ equal to unity at 5°C ., where R and R_0 are respectively the rates of reaction in the presence and absence of the enzyme.

(b) Estimation of Zinc

Zinc content of the preparation was determined polarographically by the method described by Wood and Sibly (1950).

(c) Preparation of Extracts for Estimation of Activity

Samples of leaves, each of 5 g. fresh weight, were ground finely in a mortar for 2 min. with acid-extracted sand together with 10 ml. freshly prepared cysteine-buffer solution. A 0.2M mixed phosphate buffer (pH 6.8) containing equimolecular amounts of KH_2PO_4 and Na_2HPO_4 was prepared on each occasion by diluting stock solutions of the salts of molar concentration with CO_2 -free distilled water. The final buffer was prepared by mixing 2 parts of the 0.2M phosphate buffer with 3 parts of cysteine solution so that the final concentration of cysteine was 0.01M. The pH of this solution was 6.6. After grinding, the resultant brei was kept at 2°C . until estimations of activity had been performed.

In our experience any leaf extract, even at 2°C ., without addition of cysteine during grinding lost its activity rapidly. (Table 1.)

Activity of the extract was also influenced by the method of disintegration of the leaf material. Disintegration for 2 min. in a Waring Blendor packed with ice reduced the activity of the brei to approximately one-half that obtained by grinding in a mortar for the same time: e.g. aliquots of material ground in the Waring Blendor had a range of activity at 5°C . from 10 to 15 enzyme units per ml., whilst the same number of aliquots ground with sand in a mortar gave a range of activities from 20 to 25 enzyme units per ml. at the same temperature.

These results after disintegration for 2 min. were consistently reproducible. Loss of activity, variable in amount, occurred when grinding in a mortar was prolonged, as for example when large amounts of leaf material were used. For this reason, when preparing large amounts of extract for purification of the enzyme, the material was disintegrated for 2 min. in a Waring Blendor cooled with ice. At the same time the loss of activity involved in this procedure was measured by comparing the activity of an appropriate aliquot of the extract with that of 5 g. fresh material ground in a mortar.

Extracts as prepared above were inactivated when dialysed against distilled water; it was found essential to dialyse the extract against dilute cysteine solution to avoid loss of activity.

TABLE 1
EFFECT OF 0.01M CYSTEINE ON ACTIVITY OF LEAF BREI STORED AT 2°C.

Time after Grinding (hr.)	Without Cysteine		With Cysteine	
	Activity (E.U./0.5 ml. extract)	Loss (%)	Activity (E.U./0.5 ml. extract)	Loss (%)
1	3.17	0	3.17	0
2	2.3	27	—	—
4	1.7	46	—	—
5	0.3	90	3.17	0
24	0	100	3.17	0

All measurements at 5°C.

III. PURIFICATION OF CARBONIC ANHYDRASE

A modification of the method of Keilin and Mann (1940) was used in purification of the enzyme.

Fresh leaves of spinach beet (*Beta vulgaris* var. *cicla*) were disintegrated for 2 min. with ice-cold 0.01M cysteine solution in a Waring Blendor cooled with ice and the mixture filtered through gauze to remove uncrushed debris. To this brei was added slowly sufficient 95 per cent. ethanol to bring the final concentration to 30 per cent., the temperature being kept near 0°C. To this mixture one-tenth of its volume of chloroform was added and the whole shaken for about 1 min. The mixture was then centrifuged at 1,700g for 10-15 min. This resulted in the formation of three layers, the lowest being chloroform, which contained all the chlorophyll and some fats; above this occurred a sludge of greenish white, coagulated cell constituents, partially protein in nature, and above this a yellow, translucent solution, which contained approximately 85 per cent. of the original enzyme activity. This solution—the “alcohol-chloroform extract”—when decanted was extremely stable, no loss of enzyme activity being observed after 27 days storage at 2°C. The stability of this enzyme preparation was in contrast with more highly purified preparations described later. Small amounts only were prepared on each occasion; in

general, 1 kg. leaf material and 850 ml. 0.01M cysteine solution were used, yielding about 800 ml. of leaf brei and about 1,150 ml. of "alcohol-chloroform extract."

Addition of lead acetate or of acetone to the "alcohol-chloroform extract" caused complete inactivation of carbonic anhydrase in the extract and for this reason these substances could not be used for purification (cf. Keilin and Mann 1940).

Willstätter's (1920) alumina C γ cream was added slowly with stirring to the "alcohol-chloroform extract," the solution allowed to stand for 5-10 min. and then centrifuged at 1,700g for 10 min.

The amount of alumina cream must not be less than 800 mg. alumina/100 ml. extract. As shown in Figure 1, lower concentrations of alumina resulted in incomplete adsorption of enzyme. These results were obtained by adding varying amounts of alumina to 100 ml. of extract and measuring the activity of both supernatant solution and the eluate from the alumina precipitate. Ca₃(PO₄)₂ used as an adsorbing agent by Meldrum and Roughton (1934) and by Keilin and Mann (1940) was as effective as but no better than alumina C γ ; 3.0 g. Ca₃(PO₄)₂ per 100 ml. extract adsorbing the enzyme completely.

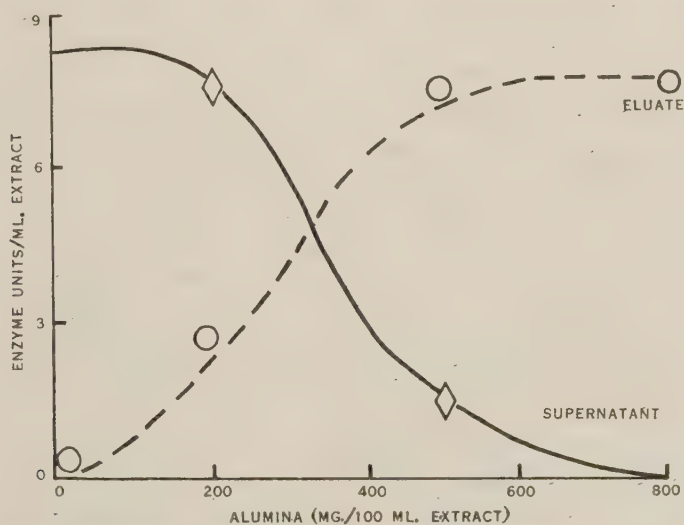


Fig. 1.—Carbonic anhydrase activity (E.U./ml. extract) absorbed by different amounts of alumina C γ (mg./100 ml. extract).

Centrifugation caused the alumina to be concentrated into a cake, which after discarding the supernatant solution, was broken up and washed two or three times with 0.1M Na₂HPO₄ to elute the enzyme. Approximately 40 ml. of phosphate solution were required for each litre of original "alcohol-chloroform extract."

To the combined eluates sufficient saturated ammonium sulphate solution was added to give a 50 per cent. saturated solution. After standing for 2 hr.

at 2°C. the precipitate was centrifuged off and dried in a vacuum desiccator. Further addition of ammonium sulphate to give 100 per cent. saturated solution brought down another precipitate which was dried in a similar manner but contained only very small activity. The solution after precipitation possessed no carbonic anhydrase activity.

The dried precipitate from 50 per cent. saturated ammonium sulphate lost its activity slowly after the first 2 or 3 days even in a vacuum desiccator (see Table 2). Activities of the precipitates from 50 per cent. saturated ammonium sulphate were consistently reproducible with different batches of leaf material but further purification has not been successful.

TABLE 2
LOSS OF CARBONIC ANHYDRASE ACTIVITY OF DRIED PARTIALLY PURIFIED ENZYME
IN A VACUUM DESICCATOR AT 15°C.

Days after Preparation	Activity (E.U./mg.)	Mean Loss (%)
1	2.88	0
2	2.88	0
3	2.0	30
6	1.1	62
7	0.66	70

Conditions of estimation: 4 mg. enzyme preparation in 2 ml. 0.2M phosphate buffer at pH 6.8; temperature of estimation, 5°C.

The precipitate obtained by 50 per cent. saturation with ammonium sulphate was dissolved and dialysed against distilled water until free from salt (48 hr.). Dialysis against distilled water caused a loss of 75 per cent. of weight of the original precipitate material but in all cases caused complete inactivation of the enzyme. Dialysis against 0.01M cysteine solution caused a similar reduction in weight and a marked, though variable, decrease in activation of the enzyme. The fragility of carbonic anhydrase from plant sources compared with that from animals will be discussed later. In calculating the activity of the material precipitated by ammonium sulphate in Table 3, measurements were taken before dialysis and activity expressed on the basis of weight of product after dialysis.

The enzyme was inactivated only in crude leaf extract in the absence of cysteine; at later stages of purification, including the final product, the same activity was obtained in the presence and absence of cysteine.

In Table 3 the increase in activity of the preparations at various stages of purity is shown and, for comparison, figures obtained by Keilin and Mann (1940) for analogous stages in purification of the enzyme from animal sources.

IV. ZINC CONTENT

At all stages the preparations contained zinc. The mean content of the dried preparation after precipitation with 50 per cent. ammonium sulphate was 0.014 per cent. zinc. On the basis of dry weight after dialysis this is equivalent to 0.056 per cent. zinc.

V. PROPERTIES OF THE PARTIALLY PURIFIED ENZYME

(a) *Temperature*

The enzyme is heat-sensitive. Complete inactivation of the 50 per cent. $(\text{NH}_4)_2\text{SO}_4$ precipitate in phosphate buffer of pH 6.8 resulted in 5 min. at 55°C ., without obvious coagulation, and 5 min. at 50°C . caused 85 per cent. inactivation.

TABLE 3
CARBONIC ANHYDRASE ACTIVITIES OF PREPARATIONS AT VARIOUS STAGES OF PURITY

	Plant Source Activity			Animal Source Activity* 15°C . A†
	5°C .	15°C .		
Plant extract	0.58	0.29	Erythrocytes	14.3
"Alcohol-chloroform extract"	0.80	0.40	"Alcohol-chloroform extract"	133
Phosphate eluent	33	18		
Purest $(\text{NH}_4)_2\text{SO}_4$ ppt.			Purest final product	2,200
50% saturated	10	6		
100% saturated	0.5	—		

* Activities in E.U./mg. dry wt. of preparation. Temperatures given are those at which estimations were carried out.

† Erythrocytes from defibrinated ox blood. Data of A from Keilin and Mann (1940).

(b) *Activity in Relation to pH*

At all stages of preparation the pH was within the range 5.8-7.0. Beyond these limits activity was lost rapidly. Samples of the enzyme preparation precipitated with 50 per cent. ammonium sulphate were dissolved in 0.3 ml. of buffer solutions in the reaction flasks and stood for 30 min. at 15°C . The activity was then determined at 5°C .

The pH, composition, and concentrations of the buffers used were as follows: pH 1.04, 0.1M HCl; pH 1.55, 0.023M citric acid *plus* 0.046M NaOH *plus* 0.2M HCl; pH 4.96, 0.1M citric acid *plus* 0.2M NaOH; pH 6.60, 0.086M Na_2HPO_4 *plus* 0.114M KH_2PO_4 ; pH 8.05, 0.0045M NaOH *plus* 0.05M H_3BO_3 *plus* 0.05M KCl; pH 9.30, 0.0294M NaOH *plus* 0.05M H_3BO_3 *plus* 0.05M KCl; pH 11.9, 0.1M NaOH. Activities in relation to pH are shown in Table 4.

(c) *Effect of Inhibitors*

The effects of various inhibitors on carbonic anhydrase activity are described below. In all cases the enzyme preparation used was that precipitated by 50 per cent. $(\text{NH}_4)_2\text{SO}_4$: 4 mg. of this preparation was dissolved in 2 ml. 0.2M phosphate buffer of pH 6.8. To this solution 0.2 ml. of a solution containing the inhibitor was added, both solutions being kept at 5°C . The solu-

tion of inhibitor contained sufficient of the latter to give the final concentrations indicated below. Estimations of enzyme activity were made at 5°C. 2-3 min. after addition of the inhibitor.

General.—General enzyme poisons inhibited the activity of carbonic anhydrase. Mercuric chloride caused 100 per cent. inactivation in 1.0×10^{-4} M solution. Merthiolate (sodium ethyl mercurithiosalicylate) gave 100 per cent. inhibition with 5.0×10^{-5} M solution.

Metal-protein inhibitors.—Both KCN and KCNS caused inhibition to approximately the same extent, 1.0×10^{-3} M solutions causing practically no inhibition whilst 1.0×10^{-2} M solutions caused about 65 per cent. inhibition.

NaN_3 was more effective as an inhibitor than KCN. 1.0×10^{-2} M solution caused 100 per cent. inhibition and 1.0×10^{-3} M solution 70 per cent. inhibition.

With Na_2S , a 1.0×10^{-3} M solution caused about 5 per cent. inhibition whilst 1.0×10^{-2} M caused 70 per cent. inhibition.

TABLE 4
ACTIVITY OF CARBONIC ANHYDRASE AFTER 30 MIN. AT 15°C. AT THE pH INDICATED

Buffer	pH	Activity (as % of activity at pH 6.6)
HCl	1.04	12
Citrate — HCl	1.55	27
Citrate — HCl	4.96	27
Phosphate	6.60	100
NaOH-borate	8.05	100
NaOH-borate	9.30	35
NaOH	11.90	12

Activity measured at 5°C. Ionic concentrations of buffers used are indicated in the text.

Sulphonamides.—Sulphanilamide solution at 1.0×10^{-3} M caused no inhibition and a 1.0×10^{-2} M solution less than 5 per cent. inhibition.

Sulphydryl inhibitors.—In view of the role of cysteine in preserving activity of crude enzyme solutions the effect of various sulphydryl inhibitors was investigated.

Sodium iodoacetate caused no inhibition in 1.8×10^{-2} M solution. However, this compound, an alkylating agent, does not necessarily react with all -SH groups present and often reacts with amino groups.

Mercaptide-forming compounds (e.g. *p*-chlormercuribenzoate and arsenicals) have a much greater affinity for -SH groups, combining with the protein to give reversible compounds that dissociate readily on addition of another thiol compound with greater affinity for the reagent, e.g. cysteine or reduced glutathione. The degree of reactivation of an enzyme inhibited by a mercaptide-forming reagent is determined mainly by the affinity of the enzyme and the thiol for the inhibitor. Generally, the higher the concentration of the thiol compound the greater the degree of reactivation.

p-Chlormercuribenzoate caused complete inhibition in 1.0×10^{-4} M solution and inhibiting effects were apparent even at dilutions of 2.2×10^{-6} M (Table 5).

TABLE 5
INHIBITION OF CARBONIC ANHYDRASE ACTIVITY BY *p*-CHLORMERCURIBENZOATE

Concentration of Inhibitor	Percentage Inhibition
1.0×10^{-4} M	100
4.3×10^{-5} M	97
2.2×10^{-5} M	90
4.3×10^{-6} M	40
2.2×10^{-6} M	24

Conditions of experiment: 4 mg. enzyme preparation in 0.2M phosphate buffer at pH 6.8; estimations made 2 min. after addition of inhibitor; temperature 5°C.

Reactivation of the poisoned enzyme by reduced glutathione is shown in Table 6, addition of the glutathione taking place 2-3 min. after addition of the *p*-chlormercuribenzoate to the enzyme solution.

TABLE 6
REACTIVATION OF *p*-CHLORMERCURIBENZOATE-INHIBITED CARBONIC ANHYDRASE BY GLUTATHIONE

Concentration of <i>p</i> -Chlormercuribenzoate	Percentage Inhibition	Concentration of Glutathione	Ratio Conc. Reactivator Conc. Inhibitor	Percentage Reactivation
1.0×10^{-4} M	100	1.6×10^{-3} M	16	0
4.3×10^{-5} M	100	1.6×10^{-3} M	37	84
2.2×10^{-5} M	87	1.6×10^{-3} M	73	100

Conditions of experiment: 4 mg. enzyme preparation in 2 ml. 0.2M phosphate buffer at pH 6.8; estimations of inhibition made 2 min. after addition of inhibitor; glutathione added 2 min. after addition of inhibitor; temperature 5°C.

The effect of sodium arsenite on inhibition of carbonic anhydrase was similar to that of *p*-chlormercuribenzoate. 100 per cent. inhibition was obtained by 5×10^{-3} M solution of arsenite. Cysteine at 5×10^{-2} M gave 20 per cent. reactivation and 1.0×10^{-1} M cysteine gave 95-100 per cent. reactivation of the arsenite-poisoned enzyme.

VI. SULPHYDRYL GROUPS IN THE PARTIALLY PURIFIED ENZYME

Carruthers (1947) developed a polarographic method for estimating the amounts of certain proteins containing -SH groups (e.g. cytochrome *c*, ascorbic acid oxidase, tyrosinase) by the catalytic wave they developed in a solution of ammonium chloride, ammonium hydroxide, and hexamino-cobaltic chloride. We confirmed that only amino acids containing -SH groups gave this wave. The partially purified products used by us, prepared with and without cysteine in the initial treatment, all gave a wave similar to that for cysteine, gluta-

thione, and cytochrome *c* (Fig. 2). We concluded, therefore, that our product contains sulphydryl groups.

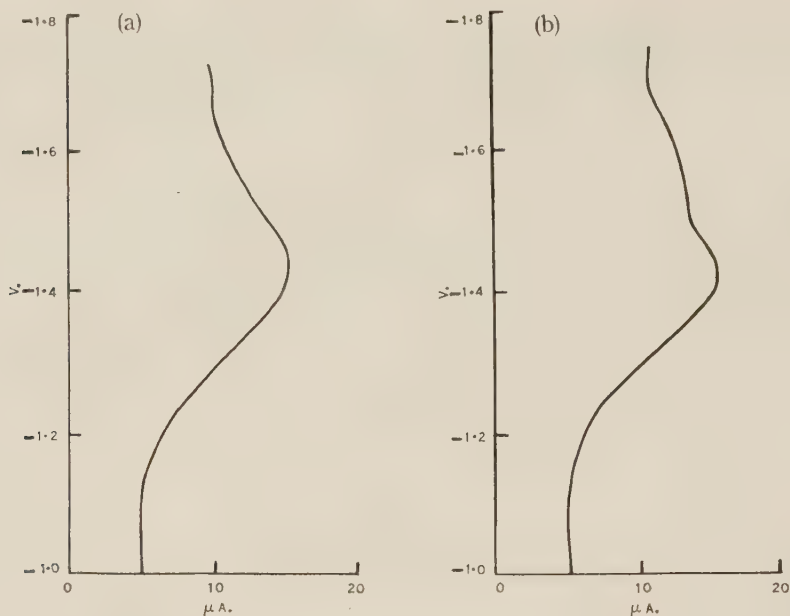


Fig. 2.—Polarographic tracing (V./μA.) of (a) cytochrome *c* (10 g./ml.) and (b) carbonic anhydrase preparation (0.1 mg./ml.). The similarity of the tracings is due to the presence of sulphydryl groups.

VII. DISCUSSION

The stability of carbonic anhydrase in crude leaf extracts is dependent upon the presence of certain reducing substances. Day and Franklin (1946) found that activity of such extracts was quickly lost but was retained in part by addition of stannous chloride. Bradfield (1947) used cysteine instead of stannous chloride to preserve activity. This effect of cysteine in preserving activity in crude leaf extracts has been confirmed by us and rate of loss of activity in its absence measured.

Apparently the presence of a reducing substance is necessary only in crude leaf extracts. The enzyme is stable in the "alcohol-chloroform extract" and in the phosphate eluate from alumina in the absence of cysteine, and activities of these solutions are the same whether measured in the presence or absence of cysteine. The enzyme preparation obtained by precipitation with 50 per cent. ammonium sulphate is more stable than the crude leaf extract but less stable than the enzyme in the two foregoing extracts; the activity of this preparation is not affected by the presence of cysteine. As described below carbonic anhydrase probably contains sulphydryl groups; cysteine and stannous chloride apparently protect the enzyme from oxidation by enzymes present in the crude leaf extract.

The amount of enzyme in leaf tissues is small compared with that in animal tissues (cf. Table 3). If the most active preparations obtained during purification of extracts from plant and animal sources are compared (the phosphate eluate from plant extract and Keilin and Mann's purest preparation) the increase in activity relative to that of the initial extracts is of approximately the same order in both cases viz. 50-60 times in plant material and 60-150 times in Keilin and Mann's preparation. However, activities (E.U./mg.) of the plant preparations are very low compared with those from animal sources at all stages of purification.

Purification of the plant enzyme is more difficult than that from animals, not only because of its low concentration in the tissues, but also because of its greater fragility.

Inactivation of carbonic anhydrase in crude leaf extract occurs in the absence of cysteine and during dialysis against distilled water. In the "alcohol-chloroform extract" complete inactivation is brought about by addition of lead acetate and of acetone. Furthermore, it may be seen from Table 3 that addition of 50 per cent. saturated $(\text{NH}_4)_2\text{SO}_4$ to the solution eluted by phosphate from alumina caused marked reduction in activity. Investigation showed that addition of 0.15M $(\text{NH}_4)_2\text{SO}_4$ to the "alcohol-chloroform extract" caused 15 per cent. inhibition of the original activity. The precipitate produced by addition of 50 per cent. $(\text{NH}_4)_2\text{SO}_4$ was inactivated by dialysis.

The procedures mentioned in the last paragraph do not decrease activity of the purified enzyme from animal sources. These differences in behaviour suggest the possibility that the enzyme from plant sources may be different from that from animal sources.

This possibility becomes more definite when the effects of inhibitors on enzymes from the two sources are compared. In our partially purified preparations from plants, KCN, KCNS, and Na_2S caused practically no inhibition in $1.0 \times 10^{-3}\text{M}$ solutions, and about 65 per cent. inhibition in $1.0 \times 10^{-2}\text{M}$ solutions. NaN_3 was more effective as an inhibitor than the foregoing compounds; $1.0 \times 10^{-2}\text{M}$ solution caused 100 per cent. inhibition and $1.0 \times 10^{-3}\text{M}$ solution 70 per cent. inhibition. Bradfield (1947) also reported that inactivation of the enzyme in crude leaf extracts was brought about only by high concentrations of cyanide and azide.

It is apparent that these substances are less effective as inhibitors of carbonic anhydrase from plant sources than is the case with the animal enzyme. Meldrum and Roughton (1934) found that $1.25 \times 10^{-3}\text{M}$ KCN caused complete inhibition of their partially purified enzyme and also of that in crude ox blood. Keilin and Mann (1940) found that concentration of KCN as low as $4.0 \times 10^{-6}\text{M}$ caused 85 per cent. inhibition of activity of their purified enzyme.

Even more marked is the contrast in behaviour of carbonic anhydrase from plant and animal sources towards sulphanilamide. Bradfield (1947) stated that sulphanilamide was ineffective in causing inactivation of the enzyme in crude leaf extracts. Sulphanilamide in $1.0 \times 10^{-3}\text{M}$ solution caused no inactivation and $1.0 \times 10^{-2}\text{M}$ solution less than 5 per cent. inhibition of original

activity of our partially purified product. With carbonic anhydrase from animal sources Mann and Keilin (1940) showed that the enzyme was strongly inhibited by sulphanilamide which they claim is highly specific for animal carbonic anhydrase. They showed that concentrations of 2×10^{-5} M sulphanilamide caused almost 100 per cent. inhibition of activity and concentrations as low as 2×10^{-6} M more than 50 per cent. inhibition of the original activity.

In view of the relative insensitivity of the plant enzyme to the foregoing inhibitors the possibility that the plant enzyme is not a metal-protein cannot be overlooked. However, this insensitivity could have been caused by the inhibitors combining with impurities (e.g. heavy metals) more readily than with the enzyme. Polarograms showed the presence of copper, as well as zinc, in our preparations and it is possible that other heavy metals were also present.

Wood and Sibly (1950) showed that zinc was not removed from crude plant extracts of oat leaves by dialysis against distilled water. All our preparations contained zinc, the precipitate with 50 per cent. ammonium sulphate contained 0.014 per cent. zinc, and the inactive product after dialysis 0.056 per cent. zinc. The purest preparation of Keilin and Mann from animal sources contained 0.31-0.33 per cent. zinc. In the absence of criteria that our preparation was a homogeneous protein, comparison with regard to zinc content between the two enzyme preparations cannot be made, nor a decision reached whether zinc is an essential constituent of the enzyme.

Since cysteine and stannous chloride preserve carbonic anhydrase activity in crude leaf extracts, Bradfield (1947) suggested that the plant enzyme shows greater dependence on sulphydryl groups than does the animal enzyme. The marked inhibition of activity of our enzyme preparation caused by low concentrations of mercaptide-forming compounds such as *p*-chlormercuribenzoate and sodium arsenite, and the reversal of these inhibitions by thiol compounds are evidence for the presence of sulphydryl groups in the enzyme. This view is supported by polarographic evidence for the presence of such groups in our partially purified product. We know of no evidence that carbonic anhydrase from animal sources is inhibited by mercaptide-forming compounds.

In view of the marked differences in behaviour of the enzyme preparation from plant tissues compared with that from animal sources it is suggested that plant carbonic anhydrase is a different enzyme from that occurring in animal tissues.

VIII. REFERENCES

- BRADFIELD, J. R. G. (1947).—*Nature* **159**: 467.
DAY, R., and FRANKLIN, J. (1946).—*Science* **104**: 363.
CARRUTHERS, C. (1947).—*J. Biol. Chem.* **171**: 641.
KEILIN, D., and MANN, T. (1940).—*Biochem. J.* **34**: 1163.
MANN, T., and KEILIN, D. (1940).—*Nature* **146**: 164.
MELDRUM, N. V., and ROUGHTON, F. J. W. (1934).—*J. Physiol.* **80**: 113.
NEISH, A. C. (1939).—*Biochem. J.* **33**: 300.
WILLSTATTER, R. (1920).—*Liebig's Ann.* **422**: 72.
WOOD, J. G., and SIBLY, PAMELA M. (1950).—*Aust. J. Sci. Res. B* **3**: 14.

INFLUENCE OF OXYGEN CONCENTRATION ON THE REDUCTION OF NITRATE BY A *PSEUDOMONAS* SP. IN THE GROWING CULTURE

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Summary

A method is described whereby oxygen tension in growing cultures of microorganisms may be assessed with reasonable accuracy at any time by means of a recording polarograph.

The method applied to a study of nitrate decomposition by a species of *Pseudomonas*, using unadapted cells as an inoculum, reveals that no apparent reduction occurs while oxygen is present in solution.

A method is also outlined for the rough assessment of substrate deficiency in cultures.

I. INTRODUCTION

Meiklejohn (1940) has investigated the effect of aeration on the reduction of nitrate, and she observed that reduction occurred under "aerobic" conditions in aerated cultures. In view of Stickland's (1931) observation on the effect of oxygen on the function of the enzyme nitratase, she was led to the conclusion that another enzyme not sensitive to oxygen was responsible for nitrate reduction under aerobic conditions. This point has been disputed by Sacks and Barker (1949) on the grounds that Meiklejohn's aeration procedure was incorrect, inferring that the rate of oxygen uptake in portions of the culture fluid exceeded its rate of solution, and hence the steady state between the gas and liquid phases had not been achieved.

Using a constant mass of adapted cells, Sacks and Barker showed by manometric methods that the enzymes involved in the reduction of nitrate and nitrite were both sensitive to oxygen, and that the rate of nitrate reduction in air is 29 per cent. of that under anaerobic conditions, whilst the corresponding value for nitrite reduction was 25-35 per cent. A constant cell mass could not be expected to exist for any more than a momentary period in growing cultures. To study the effect of oxygen concentration on nitrate reduction in growing cultures it is necessary to know not only the actual oxygen concentration in the medium in relation to nitrate concentration but also the availability of oxidizable substrate at different time intervals during growth. Desaturation of the oxidative enzyme systems with substrate will reduce the ability of the organism to utilize both the oxygen and nitrate. This could give the impression that a high oxygen concentration coupled with lack of nitrate reduction indicated inhibition of nitrate reduction by oxygen, when, in effect, conditions were unsuitable for the reduction of either.

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As far as the authors are aware, there is no evidence to indicate that organisms are capable of utilizing oxygen from air without its prior solution in the supporting medium, whether the latter be liquid or solidified with agar. It seems reasonable to assume therefore that the important value to be determined is the actual oxygen concentration in solution.

All previous workers, including Meiklejohn (1940) and Sacks and Barker (1949) have employed an indirect method of assessing the oxygen status of the medium. All of the work has been based on the assumption that, by continuous passage of a gas mixture through a solution, equilibrium between the concentration of gas in the gas and liquid phases could be achieved. In a sterile fluid this must be the case but in a fluid containing actively metabolizing cells a second system is introduced, which is continually depleting the solution of oxygen, and as the mass of cells increases the rate at which this depletion occurs increases. Hence aeration of a solution at a constant rate, sufficient to maintain a steady state under sterile conditions, may fail to meet the demand of the deoxygenating system, resulting in the complete removal of oxygen from the solution.

To attack the problem directly it was necessary to develop a technique that would permit the determination of oxygen at any chosen time in a growing culture that was being constantly aerated, and at the same time to determine the availability of substrate and also nitrate and nitrite concentration. The apparatus and methods for achieving this are described in the following sections.

TABLE 1
OXYGEN CONCENTRATIONS IN PEPTONE YEAST EXTRACT BROTH UNDER VARYING
DEGREES OF AERATION AT 25°C.

Stirring Rate (r.p.m.)	O ₂ Concentration (p.p.m.)
500	8.1
300	8.0
200	8.0
100	7.9
Unstirred	7.2

II. APPARATUS AND ANALYTICAL METHODS

(a) *Apparatus and Technique for Determination of Oxygen in Aerated Culture Fluids*

As will be evident from Table 2, 500 r.p.m. series, the rate of deoxygenation at times exceeded 8 p.p.m. per min. The steady state concentrations of oxygen in the medium at the temperature and the stirring rates employed are set out in Table 1. The low value obtained in the unstirred solution is due to the slow uptake of oxygen by components of the medium. If placed in a sealed vessel the oxygen concentration will gradually fall to zero.

Oxygen readings had therefore to be obtained within 10 seconds or less to be of any significance. The only method that presented any possibility of achieving this object was that employing the dropping mercury electrode (Skerman and Millis 1949). This method could determine 0.06 p.p.m. of oxygen with the apparatus employed. Quantities smaller than this are referred to in the text as "zero" oxygen concentration.

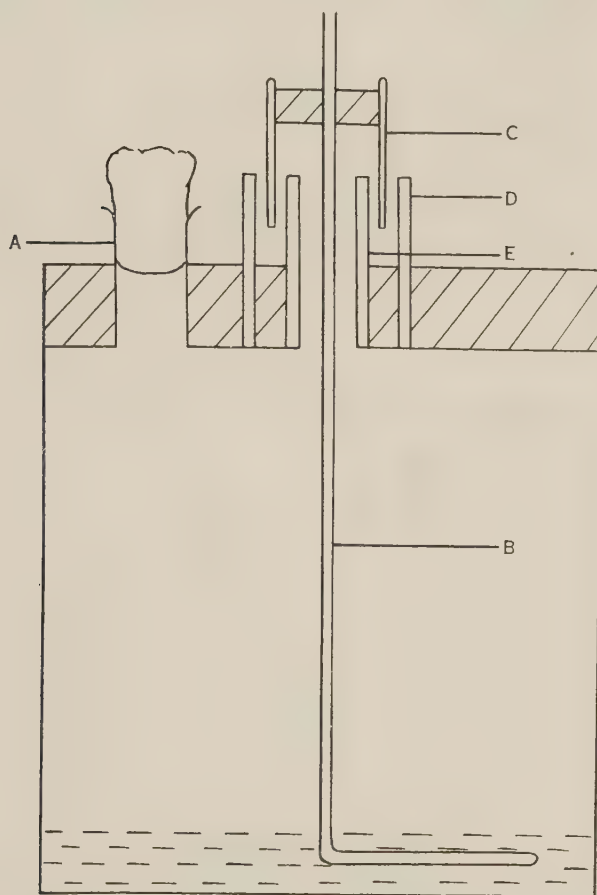


Fig. 1.—Vessel used in aeration of cultures. The vessel is of glass sealed with a rubber bung fitted with an inoculation inlet *A* and two metal cylinders *D* and *E*. The bent glass rod *B* is used for stirring. Sterility is maintained by the glass cylinder *C* spinning between the metal cylinders *D* and *E*.

The normal stationary electrode is useless in turbulent solutions unless the movement of the solution is constant both in speed and direction. It cannot, therefore, be introduced directly into a vigorously aerated solution. A possible alternative was the rotating platinum micro-electrode. The present authors

attempted to incorporate such an electrode in a stirring device used for aeration without success and eventually reverted to the following method, which provided readings that represented the oxygen status of the culture within 3 sec. of sampling.

(i) *Apparatus*.—The main components of the apparatus were a Cambridge photographic recording polarograph; a constant-speed stirring machine with the stirring rate adjustable at 100, 200, 300, 400, and 500 r.p.m.; a specially designed culture vessel (Fig. 1), a "Hyvac" pump, and a polarographic cell maintained in a constant-temperature water-bath.

When 75 ml. of the medium employed was placed in the culture vessel, a 2 cm. depth of fluid resulted. Aeration was achieved by stirring with the glass rod. No provision was made for replenishment of fresh air in the jar. Experimental evidence suggests that the supply of free air is adequate. In any case it is not necessary since correlation is made between nitrate reduction and the actual oxygen present in the solution, not with that in the gas phase.

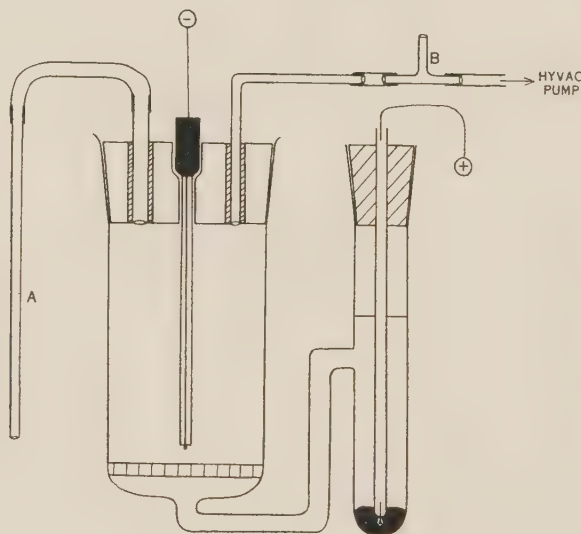


Fig. 2.—Polarographic cell assembly used for sampling and measuring oxygen concentration.

(ii) *Sampling*.—Reference to Figure 2 will show the type of apparatus employed. The cell is the same type as that employed in previous work (Skerman and Millis 1949). Removal of the sample from the aerated flask to the polarographic cell was achieved in less than two seconds by turning on the pump, inserting the pipette A into the flask while the stirring machine was still operating, and then pressing the thumb over the outlet B. Preliminary trials showed that in 2 sec. or less the cell was filled.

To avoid admixture of air with the sample as it surged into the cell, nitrogen was blown through the opening *B* to displace all air through the pipette *A* before the sample was taken.

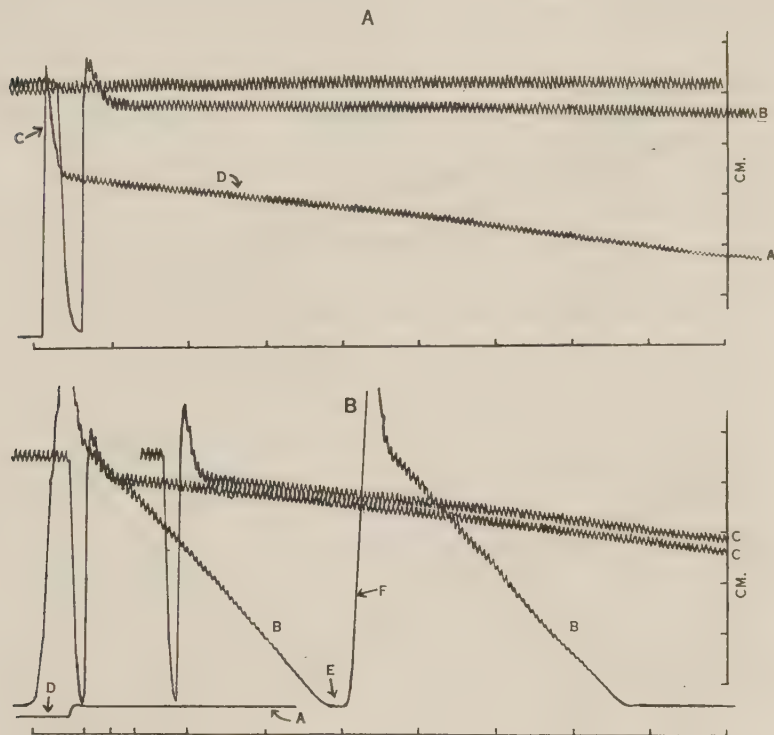


Fig. 3.—Polarograms from which figures in Table 1 were derived. In all cases the time interval (abscissa) is 57 sec. The oxygen concentration is expressed in cm. galvanometer deflection (1 cm. = 1.36 p.p.m. O_2). A, B, C, D, and E are polarograms for the 4, 8, 12, 16, and 20 hr. sampling periods respectively. For explanation see text.

(iii) *Recording of Oxygen Concentration in the Sample.*—Prior to the acquisition of a Cambridge recording polarograph, readings were made of galvanometer deflection visually. Most of the findings obtained by this method were subsequently confirmed by the recording instrument. However, where uptake rates were high or the oxygen concentration low, or both, as was often the case, accurate visual readings were impossible. The recording polarograph (see Kolthoff and Lingane 1946) overcame this difficulty. The machine was coupled with the polarographic cell in the usual manner and adjusted to record current flow at a fixed applied e.m.f. of 0.6 V. Just prior to sampling the machine was set in motion. Owing to the initial absence of an electrolyte in the cell the circuit was broken and hence the polarograph recorded the galvanometer zero reading as a black line running parallel to the abscissa (Fig. 3B, curve D).

When the culture fluid was sucked into the cell the circuit was closed with the resulting sudden deflection of the mirror galvanometer. The behaviour of the galvanometer needle at this instant depended on the existence and quantity of reducible ion in solution. With fluids devoid of oxygen and more than

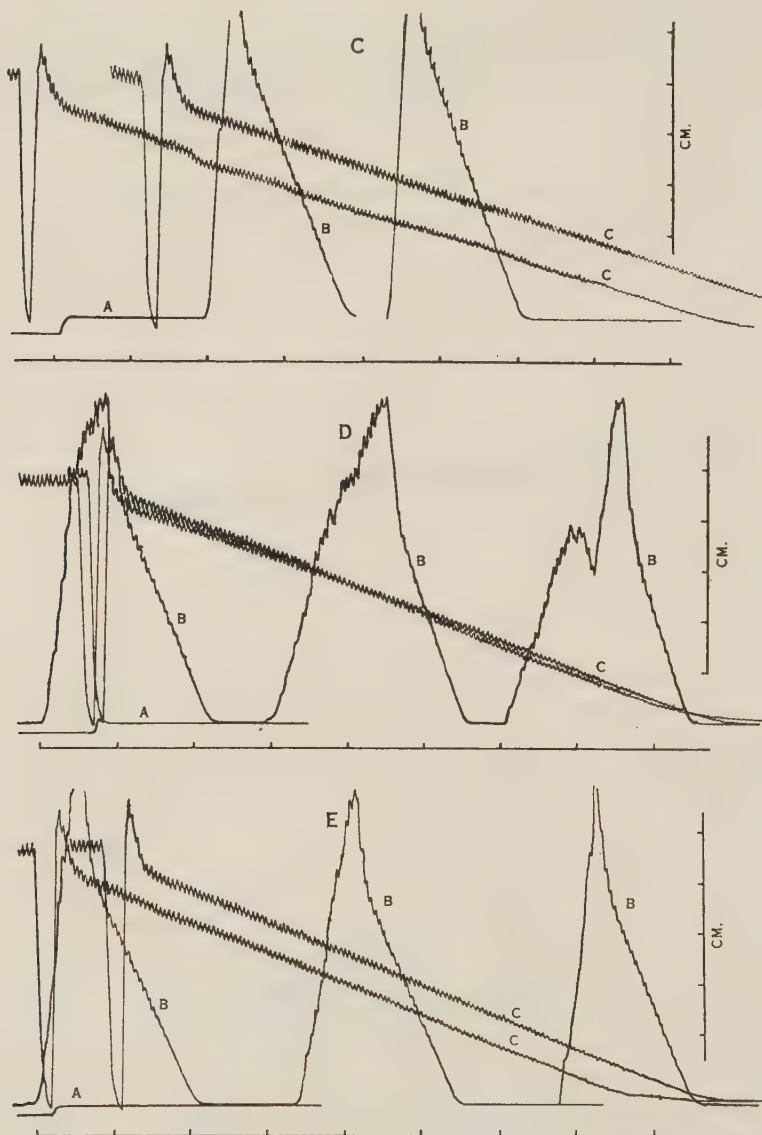


Fig. 3 (C-E).—For details see page 515.

traces of other reducible ions the galvanometer needle merely shifted position from the galvanometer zero to the residual current reading for the fixed applied e.m.f. employed (Fig. 3B, curve A). Turbulence alone would only be responsible for a very minor fluctuation in current if reducible ions were absent.

In the presence of reducible ions the turbulence of the solution caused the movement of the ions to the mercury drop by other than pure diffusion, with the result that the readings were extremely erratic and unduly high until turbulence subsided (Fig. 3A, curve C). Once turbulence subsided the current decreased at a uniform rate as the oxygen was removed from solution (Fig. 3A, curve D).

By extrapolating this slope back to zero time a reasonable assessment of the oxygen concentration at the sampling instant was obtained.

(b) Estimation of Substrate Availability

It is generally accepted that nitrate functions as an alternative to oxygen in respiration by cells containing the enzyme nitratase. The two major factors limiting its reduction are oxygen on the one hand and availability of oxidizable substrate on the other. If, after the removal of oxygen, the available oxidizable substrate should be depleted it is obvious that no further nitrate can be reduced and metabolism ceases. The present authors considered that, for the purposes of this problem, a rough assessment of substrate availability could be obtained by comparing the oxygen uptake rate of a portion of the culture itself with the oxygen uptake rate of a portion of the culture sown into a fresh sample of the same culture medium.

For example, in the absence of other governing factors, the uptake rate of 2 ml. of the culture when sown into 20 ml. of the fresh medium should be 1/11 of that of the whole population in the original culture provided the latter had plenty of readily oxidizable substrate. If on the other hand the substrate in the original culture was partially or wholly depleted then the ratio would become increasingly narrow and finally reversed as the availability of substrate was reduced to zero.

This method was therefore adopted. Where the initial oxygen concentration was large the rate of O_2 uptake was obtained from the graph (Fig. 3A, curve D). Where it was low, oxygen was blown into the sample of culture in the cell to bring the level as near to the concentration existing in the sterile medium as practicable and the uptake rate then determined (Fig. 3B, points E, F, B).

(c) Estimation of Nitrate and Nitrite

Both nitrate and nitrite yield the same colour when determined colorimetrically by the brucine method (Noll 1945). Where mixtures occur the colour densities are additive. Nitrite can be determined in the presence of nitrate by the method of Rider and Mellon (1946). In this work standard curves were prepared for nitrate and nitrite concentrations by the brucine method and for nitrite by the Rider and Mellon procedure.

In the analysis of mixtures, nitrite was estimated by the Rider and Mellon procedure and the equivalent colour density obtained for the brucine method from the standard curve. This, subtracted from the total density determined by the brucine procedure, yields the concentration of nitrate.

The method is based on the assumption that both standard curves are in accordance with Beer's Law. Actually neither was a straight line over the whole range employed. The results are not strictly accurate where high concentrations (> 300 p.p.m.) of nitrite are present. However, on test mixtures of nitrate and nitrite, the method of correction gave the values for nitrate present.

Example of correction.—Initial nitrate, density 0.420, concentration 1700 p.p.m.; final nitrate, density 0.367; nitrite by Rider and Mellon method, 460 p.p.m. In the material diluted 1/100 for the nitrate test there would be 4.6 p.p.m. of nitrite. From the nitrite brucine calibration this corresponds with a density of 0.18.

Subtracting this from the final nitrate density,
 $0.367 - 0.18 = 0.187$.

From the nitrate brucine curve this corresponds with a nitrate concentration of 650 p.p.m. The nitrate loss is therefore 950 p.p.m.

(d) *The Organism*

The organism employed was a species of *Pseudomonas* isolated from a clay loam at Dookie, Victoria.

(e) *The Culture Medium*

The culture medium employed in the study consisted of "Difco" peptone 1 per cent., "Difco" yeast extract 0.5 per cent., NaCl 0.5 per cent., KNO_3 0.2 per cent. The medium was made up in tap water and had an initial pH of 6.8. Preliminary trials had shown that the fully oxygenated medium with and without nitrate supported luxuriant growth. In the presence of the organic nitrogen the nitrate was not reduced. It was assumed therefore that the organic nitrogen was preferentially used and that the nitrate was involved under anaerobic conditions only in respiratory function.

(f) *The Inoculum*

The organism was grown on "Difco" peptone yeast extract agar slopes at 25°C . for 48 hr. The growth was suspended in sterile Ringer's solution and diluted to a density that gave a deflection of 50 mm. with the Evelyn photoelectric colorimeter, employing a 660 filter and full illumination. This approximated 10^9 cells per ml. One ml. was used to inoculate 75 ml. of medium.

III. GENERAL PROCEDURE AND RESULTS

Briefly, the general procedure adopted was to autoclave the nitrate medium in the flask, attach it to the stirring machine and run the machine at the desired speed for 24 hr. prior to inoculation to ensure saturation of the medium with air at the temperature employed (25°C .) and freedom from contamination. The medium was then inoculated and after a fixed interval of time samples withdrawn for analysis.

Varying degrees of aeration were obtained by stirring the culture at speeds of 100, 200, 300, and 500 r.p.m. The froth created, especially at 500 r.p.m. was beaten down to a great extent by the stirring rod. For each speed, determinations were made of the oxygen level; the availability of substrate, residual nitrate and nitrite; pH change, population density, viable count (8 replicates), and cell size (averaged from 20 cell measurements) at intervals of 4, 8, 12, 16, 20 hr. after inoculation. A fresh flask was assembled for each time interval. The results of these experiments are set out in Table 2.

The photographic recordings for the 100 r.p.m. series are shown in Figures 3A, B, C, D, and E.

(a) Discussion of Results

Reference to Table 2 will show that nitrate reduction did not commence before the oxygen concentration was reduced to "zero," at 100 r.p.m. The ratio in column 6, used to express the availability of substrate should theoretically be unity and remain so as long as substrate is readily available. In fact the ratio was nearer 2.0. The authors can offer no explanation for this at present. The fact that it remains constant at approximately 1.8 indicates that substrate was at no time a limiting factor.

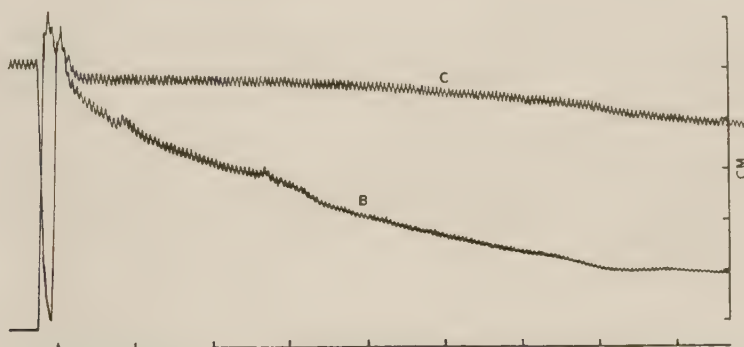


Fig. 4.—Polarogram showing rate of oxygen uptake in the original culture (curve B) and dilution culture (curve C) at 16 hr. in the 300 r.p.m. series. Abscissa, time intervals = 57 sec. Ordinate, 1 cm. = 1.36 p.p.m. O_2 .

Reference to the 200 r.p.m. figures reveals a similar state up until the 16-20 hr. period. The change in ratio in column 6 to 6.7 indicates lack of substrate in the original culture medium sufficient to maintain the enzymes in a saturated state. However, the latter was maintained sufficiently long to effect complete reduction of the nitrate.

Reference to Table 2, 500 r.p.m., reveals that, at this high speed of stirring, complete oxidation of the substrate occurred before the population was able to reduce the oxygen to "zero" concentration. No nitrate was reduced.

In the 300 r.p.m. series the oxygen concentration, owing to the increased aeration rate, did not reach "zero" until 10-12 hr. The return to practically saturation level at 16 hr., only partial reduction of nitrate, and the reduction

TABLE 2
RESULTS OF EXPERIMENTS

RESULTS OF EXPERIMENTS

Time of Sampling After Inoculation (hr.)	Oxygen Uptake Rates (p.p.m./min.)					Oxygen Uptake Rate in Diluted Culture X11 Oxygen Uptake Rate in Original Culture	Nitrate Lost (p.p.m.)	Nitrite Present (p.p.m.)	Δ pH	Density $L = 2 - \log G$	Plate Count $\times 10^3$	Average Cell Size (μ)
	Oxygen Level (p.p.m.)	Of Original Culture		Of 9.1% of Culture Inoculated into Fresh Medium								
		Initial	Reoxygenation	After	After							
Stirring Rate 100 r.p.m.												
4	4.8	0.24	—	—	0.21	—	0	0	0.1	0.0434	—	1.8×1.0
4	5.7	0.3	—	—	—	—	0	0	—	0.0362	19	1.8×1.0
8	0	—	2.4	—	0.26	1.2	0	20	0.5	0.2676	500	1.7×0.8
8	0	—	3.4	—	0.26	0.8	0	57	0.8	0.2882	100	1.7×0.8
12	0	—	5.4	—	0.69	1.4	600	90	1.0	0.557	2800	1.6×0.5
12	0	—	4.5	—	0.75	1.8	Total	280	1.2	0.545	4100	1.6×0.5
12	0	—	4.2	—	0.69	1.8	Total	460	1.0	0.512	4100	1.5×0.5
16	0	—	5.0	—	0.78	1.7	Total	0	1.2	0.699	6000	1.7×0.4
16	0	—	5.1	—	0.86	1.8	Total	0	1.4	0.716	—	1.4×0.5
20	0	—	5.0	—	0.85	1.9	Total	0	1.4	0.776	6000	1.5×0.4
20	0	—	5.0	—	0.85	1.9	Total	0	1.4	0.776	6000	1.5×0.4
Stirring Rate 200 r.p.m.												
4	6.8	0.21	—	—	—	—	0	0	0.05	0.0458	29	2.1×0.8
7	2.5	2.4	—	—	0.3	1.5	0	0	0.6	0.319	300	2.1×0.8
8	0	—	3.04	—	0.3	1.1	0	0	0.5	0.301	600	2.1×0.7
8	0	—	3.7	—	0.42	1.2	0	0	0.6	0.310	600	1.9×0.7
9	0	—	6.1	—	0.54	0.9	0	15	1.1	0.459	720	1.7×0.5
12	0	—	5.7	—	0.88	1.7	1100	380	1.2	0.699	2500	1.5×0.5
12	0	—	4.9	—	0.96	2.1	800	250	1.2	0.699	3500	1.4×0.6
16	0	—	14.2	—	1.57	1.2	1800	0	1.5	0.912	9000	1.4×0.4
							(Total)					
16	0	—	15.5	—	1.50	1.1	Total	0	1.3	0.912	10000	1.4×0.4
20	0	—	2.2	—	1.36	6.7	Total	0	1.6	0.912	9000	1.2×0.5

TABLE 2 (Continued)

Time of Sampling After Inoculation (hr.)	Oxygen Uptake Rates (p.p.m./min.)				Oxygen Uptake Rate in Diluted Culture X11 Oxygen Uptake Rate in Original Culture	Nitrate Lost (p.p.m.)	Nitrite Present (p.p.m.)	Δ pH	Density $L = 2 - \log G$	Plate Count $\times 10^6$	Average Cell Size (μ)
	Of Original Culture		Of 9.1% of Culture Inoculated into Fresh Medium								
	Initial	After Reoxygenation	Initial	After Reoxygenation							
	Stirring Rate 300 r.p.m.										
4	8.9	0.21	—	—	0	0	0	0	0.0458	28	2.0×0.8
8	8.6	0.43	—	—	0	0	0	0.5	0.382	800	2.0×0.6
10	2.8	2.1	—	—	0	0	0	1.1	0.699	4900	1.4×0.5
12	0.2	0	9.7	1.6	1.8	750	300	0.8	0.912	6000	1.3×0.5
12	0.2	0	3.2	0.69	2.2	—	460	0.7	0.912	7700	1.3×0.4
16	7.3	1.36	Not done	0.069	0.6	950	460	1.1	0.912	5000	1.5×0.5
16	5.6	0.96	Not done	0.136	1.6	900	510	1.0	0.912	8300	1.5×0.4
20	6.9	0.54	Not done	0.136	2.7	1000	460	1.3	1.000	7000	1.1×0.5
20	6.2	0.27	Not done	0.136	5.4	1150	600	1.3	1.126	9000	1.0×0.5
Stirring Rate 500 r.p.m.											
4	6.7	0.09	—	0.02	2.5	0	0	0.02	0.0605	9	1.7×0.9
4	7.7	0.18	—	0.01	0.6	0	0	0.2	0.0757	13	2.0×0.8
8	5.5	0.8	—	0.22	3.0	0	0	1.0	0.1427	200	1.9×0.7
8	5.1	1.1	—	0.26	2.6	0	0	1.0	0.2291	500	2.0×0.7
12	5.0	1.5	6.1	1.8	1.3	0	0	1.1	0.838	9500	
12	0	—	8.1	1.9	2.6	0	24	1.0	0.921	9000	
16	5.1	0.98	—	1.3	14.0	0	0	1.0	0.886	6000	1.3×0.5
16	5.8	0.77	—	1.1	16.0	0	0	1.5		11000	1.4×0.6
20	5.6	0.47	—	0.89	21.0	0	10	1.8		13000	0.8×0.5
20	8.0	0.7	—	0.98	15.0	0	14	1.0		15000	0.7×0.5

in oxygen uptake rate by the original culture strongly suggested deficiency of substrate. In a duplicate series in which the substrate concentration was doubled the oxygen concentration was reduced to "zero" and maintained there until the 20 hr. period. Nitrate completely disappeared.

The failure of the ratio in column 6 to change sharply during the 12-16 hr. period suggested that the nitrite that had accumulated acted adversely on the organism and partially inhibited oxygen uptake in the diluted culture.

Reference to Figure 3 will show that at all oxygen concentrations, both in the original (*B* curves) and diluted (*C* curves) samples, the rate of uptake of oxygen was uniform. This also applies to the 200 r.p.m. and 500 r.p.m. series.

In the 300 r.p.m. series the rate was virtually uniform until the 12 hr. period, i.e. until the appearance of nitrite. Subsequent to this the polarograms take the form shown in Figure 4.

In the original culture (curve *B*) the rate of oxygen uptake diminishes as the oxygen concentration diminishes. In the diluted sample (*C*) the rate is relatively constant for one minute and then gradually increases. This also suggested that nitrite inhibited oxygen utilization.

To test the effect of nitrite the following experiments were conducted:

(i) Cells were grown in Roux bottles on a "Difco" peptone yeast extract agar and washed off with sterile Ringer's solution. The suspension employed contained approximately 10^{10} cells per ml. One ml. of suspension was added per 10 ml. of medium in the polarographic cell. The oxygen uptake curves were recorded with the Cambridge polarograph, in the following media:

- (1) The peptone yeast extract medium alone,
- (2) The same medium plus 0.2 per cent. KNO_3 ,
- (3) The same medium plus 0.2 per cent. KNO_2 .

The result is shown in Figure 5.

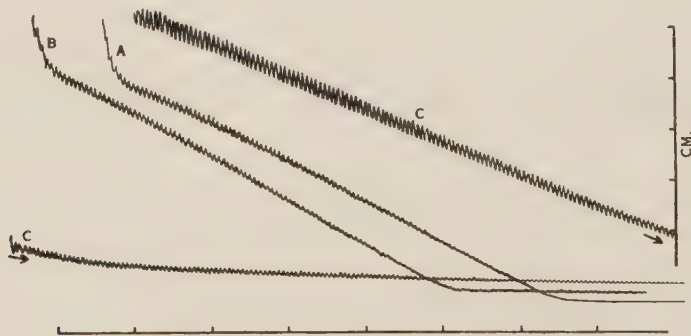


Fig. 5.—Relative rate of oxygen uptake by identical cell suspensions in A, broth alone; B, broth + nitrate; C, broth + nitrite.

There is virtually no difference between the broth alone (*A*) and broth plus nitrate (*B*). The latter was usually a shade faster than the former. With nitrite (*C*), however, the time taken to remove all the oxygen exceeded twice that for broth alone. (Note: In the recording polarograph the paper rotates on

a drum. Since curve *C* was incomplete at the end of one revolution the drum was returned to the starting point for continuation of the curve.)

(ii) Cells were grown, suspended in saline, and washed twice. Two ml. of the cell suspension were added to 16 ml. saline containing 500 p.p.m. nitrite. After varying time intervals 2 ml. of a solution containing 10 per cent. peptone and 5 per cent. yeast extract were added, the solution was mixed and then placed in the polarographic cell and reoxygenated. Oxygen uptake rates were then determined. The results are shown in Figure 6.

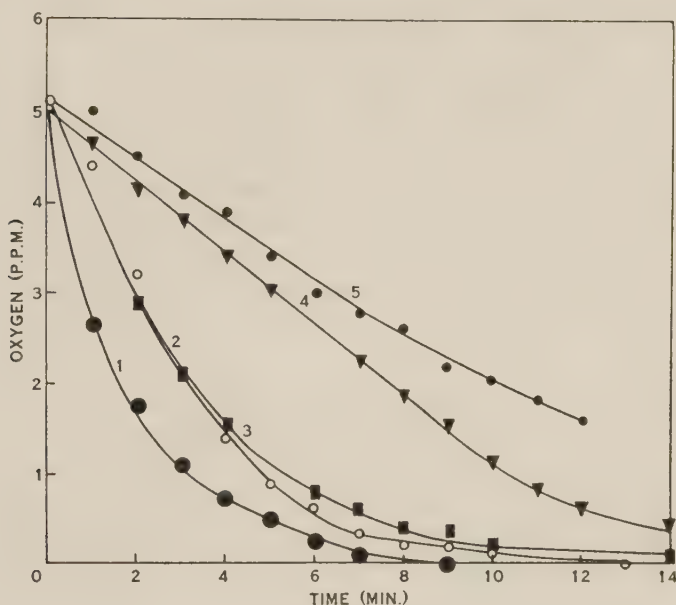


Fig. 6.—Relative rates of oxygen uptake by cells exposed to 500 p.p.m. nitrite prior to inoculation into broth. Curves 1, 2, 3, 4, 5 were obtained with cells exposed for 0, $\frac{1}{2}$, 1, 2, and 4 hr. respectively.

Curve 1 is similar to curve *B* in Figure 4. As the time of exposure increases, the effect of nitrite poisoning becomes more pronounced. This poisoning effect explains the sudden inability of the cells to utilize oxygen in the dilute samples (Table 2, column 5, 200 r.p.m.) after the appearance and accumulation of nitrite in the 300 r.p.m. series and hence explains the absence of higher ratios in column 6 (200 r.p.m.) from the 12 hr. period onwards.

IV. DISCUSSION

The work reported in this paper emphasizes the need for correlating nitrate reduction with substrate availability and oxygen concentration. Although unadapted cells were used for an inoculum, the reduction of nitrate, once the

oxygen concentration was reduced to "zero," was not long delayed. In view of the fact that "aerobic" conditions depress the formation of nitrate-reducing enzymes, one would not expect to find much, if any difference between cultures inoculated with adapted and unadapted cells. The initial population used in these experiments, 10^7 cells per ml., forms only about 2 per cent. of the cells present at the time the oxygen concentration reaches "zero." Ninety-eight per cent. of the population at this stage developed largely under conditions that would preclude enzyme formation.

According to Sacks and Barker, the nitrite-reducing enzyme(s) are not produced until the oxygen concentration is reduced to a level in equilibrium with 5 per cent. in the gas phase (approx. 2 p.p.m. at 25°C.). Even at this level they report 50 per cent. inhibition of activity. An increase in nitrite-reducing activity that would parallel a further reduction in oxygen concentration on the one hand and increase in cell mass (in growing cultures) on the other, would be expected to result in an increase in the amount of nitrite reduced in a given time interval. However, in actively growing cultures the time elapsing between the establishment of conditions satisfactory for the formation and function of the enzyme and the depletion of oxygen from the medium is so small that one could expect little, if any, nitrate reduction to occur during this period.

An important aspect of bacterial metabolism that seems to be little appreciated is the fact that an organism may use two alternative respiratory mechanisms at the same time, provided conditions are satisfactory for both. In the foregoing experiments it is obvious that oxygen must still be entering the solution and be utilized by the cells even though the oxygen concentration in solution is at a "zero" level, which permits simultaneous utilization of nitrate.

It seems possible that the so-called oxygen inhibition of nitrataase activity may be more apparent than real. Information on this point has been gained by comparing the amount of nitrate reduction occurring under various "aerated" conditions with that occurring under strictly anaerobic conditions. Exactly similar curves to those reported, for instance, by Sacks and Barker could be obtained by gradually increasing the amount of oxygen entering a culture having an initial "zero" oxygen tension. The non-aerated culture would represent the "anaerobic condition." As aeration increases, the competitive action between oxygen and nitrate would, according to Stickland's observations, result in greater utilization of oxygen in preference to nitrate. The quantity of nitrate decomposed would diminish as the amount of available oxygen was increased, whilst the concentration of oxygen in solution at all times remains at "zero."

V. ACKNOWLEDGMENT

The authors are indebted to Mr. R. Bayly for considerable technical assistance during the conduct of this work.

VI. REFERENCES

- KOLTHOFF, I. M., and LINGANE, J. J. (1946).—"Polarography." (Interscience Publishers Inc.: New York.)
- MEIKLEJOHN, J. (1946).—Aerobic denitrification. *Ann. Appl. Biol.* **27**: 558.
- NOLL, C. A. (1945).—Determination of nitrate in boiler water by the brucine reagent. *Industr. Engng. Chem. (Anal. Ed.)* **17**: 426.
- RIDER, B. F., and MELLON, M. G. (1946).—Colorimetric determination of nitrites. *Industr. Engng. Chem. (Anal. Ed.)* **18**: 96.
- SACKS, L. E., and BARKER, H. A. (1949).—The influence of oxygen on nitrate and nitrite reduction. *J. Bact.* **58**: 11.
- SKERMAN, V. B. D., and MILLIS, NANCY (1949).—The application of the polarograph to the determination of oxygen consumption rates in bacterial culture media. *Aust. J. Exp. Biol. Med. Sci.* **27**: 183.
- STICKLAND, L. H. (1931).—The reduction of nitrates by *Bact. coli*. *Biochem. J.* **25**: 1543.

STUDIES IN THE NATURAL COATING OF APPLES

I. PREPARATION AND PROPERTIES OF FRACTIONS

By F. E. HUELIN* and R. A. GALLOP*

[Manuscript received May 10, 1951]

Summary

The preparation and properties of the major fractions of the natural coating of apples are described. These include the oil, wax, ursolic acid, and "cutin" fractions. Particular attention has been given to the oil fraction, which contains unsaturated esters, and the "cutin" fraction, which gives complex hydroxy acids on saponification. The use of ammonium oxalate for separating apple skin is described.

Methods are given for quantitative determination of the coating fractions. The distribution of these fractions in the cuticle and epidermis is discussed.

I. INTRODUCTION

The natural coating of apples is of considerable importance in the physiological behaviour of the fruit as it forms the major barrier to diffusion of water vapour and other gases. Respiration and resistance to diffusion cause the internal atmosphere of the fruit to have a lower concentration of oxygen and a higher concentration of carbon dioxide than the outside air. Trout *et al.* (1942) have shown that most of the resistance to gaseous diffusion is in the skin.

Sando (1923) separated the apple skin mechanically from the flesh after soaking in dilute hydrochloric acid. He extracted the skin with light petroleum and obtained a wax, which was purified by removing coloured impurities with 80 per cent. acetone. He found a saturated hydrocarbon and a saturated secondary alcohol as major constituents of the wax. Subsequently Chibnall *et al.* (1931) identified nonacosane, heptacosane, *d*-10-nonacosanol, *n*-hexacosanol, *n*-octacosanol, and *n*-triacontanol. Most of the alcohol fraction was present in the free form. The presence of nonacosane and 10-nonacosanol was confirmed by Markley, Hendricks, and Sando (1932).

Sando (1923) extracted apple skin with ether (after previous extraction with light petroleum) and obtained a further fraction. The crude material was crystallized from alcohol and gave a melting point of 280°-282°. Subsequently Sando (1931) obtained a more highly purified product, melting at 285°, which he named ursolic acid and showed to be identical with preparations from other sources. Later workers have obtained melting points varying from 284° to 291°.

Gane (1931) extracted coating material from whole apples by immersion in ether. He separated the fractions by crystallization and distillation and

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obtained an oil in addition to the wax and ursolic acid. The oil was shown by its saponification and iodine numbers to be mainly unsaturated esters.

After extraction with solvents the residual skin is still water-repellent and retains lipoid stains. The material responsible for these properties is known as "cutin." Markley and Sando (1933) determined "cutin" by an empirical procedure. After preliminary extraction with dilute alkali and acid, the residual skin was boiled for three hours with 3 per cent. alcoholic potassium hydroxide. The loss of weight in the last treatment was taken as "cutin" originally present.

Markley and Sando (1931) followed the changes in the natural coating during maturation on the tree and subsequent storage at 0°C. The "total ether extract" (containing oil, wax, and ursolic acid) was first weighed and the ursolic acid was then determined by titration. The "oily fraction" was calculated by subtracting ursolic acid from "total ether extract." They obtained increases in the surface concentration of both fractions during maturation and storage. Subsequently Markley and Sando (1933) found that the concentration of "cutin" increased with maturity.

The present authors have made further studies of the fractions of apple skin. Particular attention was given to the oil fraction, which had been largely neglected, probably on account of its low concentration in freshly picked apples. Changes during storage were investigated, and the various fractions were usually determined by direct separation and weighing. The work was mainly concerned with the Granny Smith variety, and all results are for this variety unless the contrary is stated.

II. EXTRACTION AND PREPARATION OF FRACTIONS

The method of soaking tissue in dilute hydrochloric acid, as used by Sando (1923), has not been found very effective for separating the skin. Ammonium oxalate solution (1-2 per cent.) has been found more effective, and is less likely to alter the constituents. The ammonium oxalate solution probably dissolves a pectic layer between the epidermal and underlying cells. A solution (pH 4) containing 1.6 per cent. ammonium oxalate and 0.4 per cent. oxalic acid separated the skin of apple peelings in 48 hr. at 37°C. and 16 hr. at 50°C. The solution containing free acid gave generally better results than ammonium oxalate alone unless the apples themselves were fairly high in acidity. The separated skin was composed of cuticle and epidermis. It was washed with water and then dried in air at room temperature.

Extraction of the skin with light petroleum (50°-70°C.) in a Soxhlet apparatus removed both oil and wax fractions. The extract was evaporated and the residue dissolved in boiling acetone (about 30 ml. per g. of material) to give a somewhat turbid solution. The solution was cooled to 0°C. with stirring, when the wax separated out. After standing overnight at 0°C., the wax was filtered off and washed with a small volume of cold acetone. The wax was re-dissolved and separated from acetone twice. The combined acetone filtrates

were concentrated to a small volume and cooled to 0°C., when a small quantity of additional wax separated. The oil fraction was obtained on evaporating the final filtrate.

Subsequent extraction of the skin with ether gave the ursolic acid fraction as a light powder. Further extraction with ethanol gave only a small quantity of soluble material. The ethanol-insoluble residue was found to yield a high proportion (about 50 per cent.) of ether-soluble acids after boiling with 0.5N ethanolic potassium hydroxide for 2 hr. After saponification the ethanol solution was filtered through a sintered glass filter and the residue washed with boiling ethanol. The filtrate was evaporated to a small volume and diluted with water. A small quantity of neutral material was extracted with ether. The aqueous solution was then acidified, and the precipitated acids extracted with ether. The yield of acids obtained by this procedure can be regarded as a measure of "cutin" content, and is probably preferable to the weight loss method of Markley and Sando (1933).

The oil and wax were obtained more readily, and with less loss of oil, by extracting whole apples with boiling light petroleum for 15 min. The solvent was boiled in the bottom of a cylindrical vessel, and the apples were placed on a platform above the boiling solvent. The condensed solvent flowed back over the apples and removed the oil and wax. Successive batches of apples could be extracted with the same charge of solvent, and 1 l. of light petroleum was found adequate for 18 kg. of apples. The extract was dried with anhydrous sodium sulphate or calcium chloride and filtered before evaporation of solvent.

The combined oil, wax, and ursolic acid fractions can be obtained by extracting whole apples with boiling carbon tetrachloride for 30 min. The wax and oil can then be separated from the ursolic acid fraction with light petroleum. This method, however, has not proved very satisfactory. The ursolic acid fraction is only sparingly soluble in carbon tetrachloride. The crude extract is difficult to dry and contains a little extraneous material from the flesh, which can be removed only by re-extraction.

It is estimated that about half the total lipid or light petroleum-soluble material of the apple is in the skin. The lipid material in the flesh was extracted with ethanol, and the ethanol extract was evaporated and re-extracted with light petroleum. A typical figure for the concentration of lipid in the flesh is 0.06 per cent., hence an apple weighing 120 g. would contain about 72 mg. of flesh lipid. The concentration of lipid in the skin is about 0.5 mg. per sq. cm., which corresponds to 70 mg. per apple (of weight 120 g. and surface area 139 sq. cm.).

The insoluble "cutin" material, which gives ether-soluble acids on saponification, is almost entirely confined to the skin. The disintegrated flesh was extracted with ethanol until all the soluble material was removed. The insoluble residue gave a negligible yield of ether-soluble acids on saponification.

III. PROPERTIES OF FRACTIONS

(a) Oil

The oil is readily soluble in ethanol, acetone, ether, chloroform, carbon tetrachloride, and light petroleum. The characteristics of oil from Granny Smith apples are approximately as follows: saponification no. 110-150, acid no. 10-30, hydroxyl no. 50-70, iodine no. (Wijs) 80-140. The characteristics of oil from Jonathan and Sturmer apples come within the same range.

Saponification of the oil gave 70-85 per cent. of fatty acids, 15-30 per cent. of unsaponifiable matter, and a negligible yield of glycerol. It was found that undesirable changes could be minimized by carrying out the saponification and subsequent treatment entirely at room temperature. The oil was dissolved in 2N ethanolic potassium hydroxide and allowed to stand for 16 hr. at 20°C. in an atmosphere of nitrogen. After diluting with four volumes of water, the unsaponifiable portion was extracted with ether. The aqueous layer was then acidified and the fatty acids extracted with ether.

A sample of fatty acids was found to have the following characteristics: acid no. 173, hydroxyl no. 122, iodine no. (Wijs) 109. The hydroxyl number indicated the presence of hydroxy acids, but these did not amount to more than one-quarter of the total acids. About 75 per cent. of the acid mixture was dissolved by boiling light petroleum (30 ml. per g.), and the soluble portion had a very low hydroxy content. The insoluble somewhat tarry residue may contain products of oxidation.

The corresponding sample of unsaponifiable matter had a hydroxyl no. of 148 and an iodine no. (Wijs) of 86. Both the acid and unsaponifiable portions were highly unsaturated.

(b) Wax

The chemical composition of the wax has already been studied thoroughly by Chibnall *et al.* (1931). Samples of wax from Granny Smith apples gave the following characteristics: saponification no. 20-40, acid no. 5-7, hydroxyl no. 33, iodine no. (Wijs) 10-15. Wax from Jonathan and Sturmer apples did not differ significantly. A sample of wax from Granny Smith apples gave 10 per cent. of acids on saponification.

(c) Ursolic Acid Fraction

The ursolic acid was recrystallized from 99 per cent. ethanol and dried at 120°C. *in vacuo* for 20 hr. The mono- and di-acetyl derivatives were prepared.

Ursolic acid.—m.p. 286-8°C. (284-291°C. in literature).

Found: C, 78.9; H, 10.6.

Calculated for $C_{30}H_{48}O_3$: C, 78.9; H, 10.5.

Monoacetyl derivative.—m.p. 287-8°C. (285-296°C. in literature).

Found: C, 77.3; H, 9.8.

Calculated for $C_{32}H_{50}O_4$: C, 77.1; H, 10.0.

Diacetyl derivative.—m.p. 197-9°C. (198-200°C. in literature).

Found: C, 75.9; H, 9.5.

Calculated for $C_{34}H_{52}O_5$: C, 75.6; H, 9.6.

The presence of ursolic acid as major constituent was confirmed for Granny Smith apples.

Other constituents are present in the crude ursolic acid fraction. Acid numbers from 85 to 105 were found for the crude fraction compared with 120 for the recrystallized ursolic acid. Saponification numbers for the crude fraction varied from 120 to 150, indicating some esterification. A small amount of waxy material, soluble in light petroleum, was obtained on saponification.

(d) "Cutin"

The acids obtained by saponifying the "cutin" fraction gave the following characteristics: acid no. 150-180, hydroxyl no. 208, iodine no. (Wijs) 50. Boiling light petroleum (30 ml. per g.) dissolved only 7 per cent. of the acid mixture. Complex hydroxy acids appear to be the major constituents.

IV. DETERMINATION OF FRACTIONS

Each fraction of the apple coating was determined quantitatively by separation and weighing. The methods of separation already described are applicable to quantitative determination with certain exceptions. Separated skin is not suitable for quantitative determination of oil, as appreciable losses may occur during soaking and separation. In one test 24 per cent. less oil was obtained from separated skin than from extraction of whole apples. The wax and ursolic acid fractions can be determined in separated skin, if the apples have not been previously extracted. But if the whole apples are first extracted with light petroleum, about 13 per cent. less of the ursolic acid fraction is obtained from the separated skin. Apparently removal of the oil and wax facilitates mechanical loss of the ursolic acid fraction.

In studying changes during storage, the following methods were used for determination of coating fractions. For determination of oil, whole apples were extracted with boiling light petroleum (50°-70°C.) for 30 min. The oil and wax were separated with acetone as previously described. Reasonably accurate results were obtained by washing the wax from the first separation with a little cold acetone and avoiding the tedious re-precipitation. For determination of wax, extracts of both whole apples and separated skin were used.

The ursolic acid fraction was determined most readily by extraction of separated skin with ether after previous extraction with light petroleum. It was also extracted from whole apples with boiling carbon tetrachloride for 30 min., separated from oil and wax, and purified by re-extraction.

The "cutin" fraction was determined in the insoluble skin that had been previously used for determination of other fractions and finally extracted with ethanol to remove all soluble material. The insoluble skin was boiled with 0.5N ethanolic potassium hydroxide for 2 hr., and the acids were separated as previously described.

The results were expressed as mg. of material per sq. cm. of apple surface. To determine the surface area of a sample, the mean weight per apple

was determined separately for each size. The specific gravity of a representative sample was determined and used for calculating the volume. The surface area of a sphere of equal volume was calculated, and this area was multiplied by 1.028 to obtain the surface area of the apple. The correction factor was obtained from measurements by Mr. E. W. Hicks.

"Total fatty acids," which give a significant correlation with oil content, were determined in some cases. They were titrated after saponification of the total light petroleum extract (mixed oil and wax). A sample of about 20 apples was extracted with boiling light petroleum (50°-70°C.) for 30 min. The extract was dried with anhydrous sodium sulphate or calcium chloride, filtered, and evaporated. It was saponified by boiling under reflux with 10 ml. of 0.2N ethanolic potassium hydroxide for 45 min. Ethanol (40 ml.) was then added and the solution titrated with 0.1N hydrochloric acid. It was necessary to keep the solution warm during titration to avoid separation of wax. The titre was subtracted from the blank and the concentration of "total fatty acids" was calculated as μE per sq. cm.

V. STRUCTURE OF CUTICLE

A transverse section of the separated skin, stained with Sudan IV, is shown in Plate 1, Figure 1. The skin consists of cuticle and one layer of cells (epidermis). The uptake of the lipid stain by the cuticle is ascribed to the presence of insoluble "cutin," as all the soluble lipoids are removed before staining.

It is still uncertain whether "cutin" is composed entirely of condensed lipid material or contains cellulose in association. On soaking skin sections in 0.5N ethanolic potassium hydroxide for about two weeks at room temperature the cuticle disappeared while the cell walls of the epidermis remained intact (Plate 1, Fig. 2). It was necessary to cover the section with a collodion film before treatment to prevent loss from the slide. There was no evidence of a cellulose residue in the cuticle, though this might have been lost from the section through complete breakdown of structure.

Chemical fractionation of the residual skin gave some evidence of an association between cellulose and lipid material. The residual (ethanol insoluble) portion of the skin was first boiled with 1 per cent. ammonium oxalate solution for 1 hr. to remove pectin. The residue was soaked in cuprammonium solution (25 g. CuO in 1000 ml. 15N NH_4OH) at 37°C. for 24 hr. to remove cellulose. The remaining material was then saponified by boiling with 0.5N ethanolic potassium hydroxide for 4 hr. The residue from saponification was treated again with cuprammonium solution. The percentage loss in weight resulting from each treatment was:

Ammonium oxalate	12.0
Cuprammonium (first treatment)	2.5
Saponification with ethanolic KOH	51.3
Cuprammonium (second treatment)	24.9
Final residue	9.3

These results suggest that a large proportion of the cellulose is combined in some way with lipid material and only becomes available to the cuprammonium reagent after saponification. The cell walls of the epidermis may contain some lipid material although they do not stain strongly.

The distribution of oil, wax, and ursolic acid in the cuticle is still largely unknown. It is probable that each fraction forms a separate phase, as neither of the solid fractions is appreciably soluble in the oil at room temperature. The wax dissolves in the oil on heating but separates out on cooling.

VI. ACKNOWLEDGMENTS

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VII. REFERENCES

- CHIBNALL, A. C., PIPER, S. H., POLLARD, A., SMITH, J. A. B., and WILLIAMS, E. F. (1931).—*Biochem. J.* **25**: 2095.
GANE, R. (1931).—*Gt. Brit. Food Invest. Bd. Ann. Rep.* p. 242.
MARKLEY, K. S., HENDRICKS, S. B., and SANDO, C. E. (1932).—*J. Biol. Chem.* **98**: 103.
MARKLEY, K. S., and SANDO, C. E. (1931).—*J. Agric. Res.* **42**: 705.
MARKLEY, K. S., and SANDO, C. E. (1933).—*J. Agric. Res.* **46**: 403.
SANDO, C. E. (1923).—*J. Biol. Chem.* **56**: 457.
SANDO, C. E. (1931).—*J. Biol. Chem.* **90**: 477.
TROUT, S. A., HALL, E. G., ROBERTSON, R. N., HACKNEY, F. M. V., and SYKES, S. M. (1942).—*Aust. J. Exp. Biol. Med. Sci.* **20**: 219.

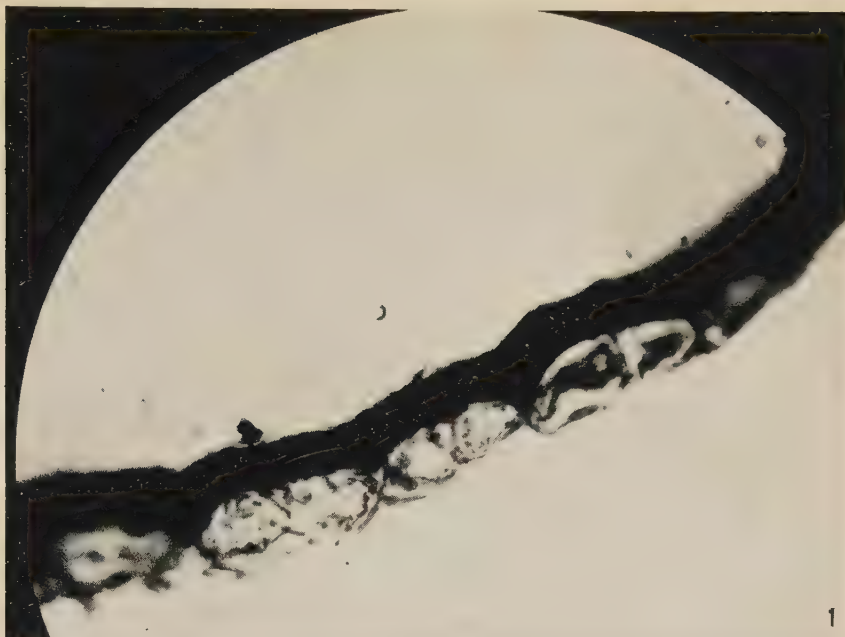


Fig. 1.—Section of separated skin (x480). The cuticle is stained with Sudan IV.
Fig. 2.—Epidermis from separated skin (x520). The cuticle was removed by ethanolic KOH.

STUDIES IN THE NATURAL COATING OF APPLES

II. CHANGES IN THE FRACTIONS DURING STORAGE

By F. E. HUELIN* and R. A. GALLOP*

[*Manuscript received May 10, 1951*]

Summary

The oil fraction of the natural coating of Granny Smith apples increased during storage and reached a maximum at 3-4 times its original concentration. The increase was reduced by "gas" storage (in 5 per cent. CO₂, 16 per cent. O₂). Later pickings had a higher oil content. The iodine number of the oil increased with increasing concentration. Smaller increases occurred in the wax, ursolic acid, and "cutin" fractions after prolonged storage.

The fatty esters of the oil fraction were produced most rapidly at the beginning of storage. Subsequently the production of these non-volatile esters declined, while the rate of volatile ester production increased.

There was no definite correlation between the oil content and the resistance of the skin to gaseous diffusion, although both increased during storage.

I. INTRODUCTION

The preparation and properties of the major fractions of the natural coating of Granny Smith apples have already been described (Huelin and Gallop 1951). Changes have been found in every fraction during storage, and the data are presented in this paper. All results refer to the Granny Smith variety.

The changes in the oil fraction are likely to have the greatest effect on the resistance of the skin to gaseous diffusion and to water loss. These changes are relatively greater than the changes in the other fractions. Moreover, the oil is the only fraction that is liquid at room temperature. Its presence may increase resistance by filling the spaces between the solid particles. For this reason the oil fraction has been studied in some detail.

II. METHODS OF ANALYSIS

The methods for determining the various fractions have already been described (Huelin and Gallop 1951). In 1945 whole apples were extracted with light petroleum and in 1947 with carbon tetrachloride. On account of the difficulties arising from extraction with carbon tetrachloride the results for 1947 are less accurate than for other years. In 1948 the separated skin was extracted first with light petroleum and then with ether. In 1949 whole apples were extracted with light petroleum for determination of "total fatty acids." The skin was subsequently separated and used for determination of insoluble "cutin."

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Iodine numbers were determined by the method of Hanus in 1945 and by the method of Wijs in 1947 and subsequent years. The method of Wijs gives considerably higher numbers.

The resistance of the skin to diffusion of oxygen was calculated by the method of Hicks from measurements of respiration and composition of internal atmosphere. The procedure is described by Trout *et al.* (1942).

All conclusions regarding changes during storage were examined for statistical significance.

III. CHANGES IN OIL CONTENT DURING STORAGE

Granny Smith apples were picked at Orange, New South Wales, on April 10, 1945, and stored at 1°C. Determinations of oil content were made after 0, 13, and 27 weeks at this temperature. Samples were also removed from 1°C. to 18°-20°C. for determination of oil content at fortnightly intervals. The iodine number of the oil and the concentration of "total fatty acids" were also determined. The results are given in Table 1 and Figure 1.

TABLE 1
CHANGES IN OIL CONTENT IN 1945
(Picked April 10, 1945 at Orange, N.S.W.)

Weeks at 1°C.	Weeks at 18°-20°C.	Oil (mg./sq. cm.)	Iodine No. of Oil (Hanus)	Total Fatty Acids (μE/sq. cm.)
0	0	0.106	50	0.265
	2	0.179	68	0.530
	4	0.247	74	0.694
	6	0.266	84	0.681
13	0	0.287	81	0.812
	2	0.277	78	0.765
	4	0.231	82	0.664
	6	0.242	81	0.726
27	0	0.302	73	0.956
	2	0.254	71	0.773
	4	0.233	72	0.654

The oil increased up to three times the original concentration at 1°C. The most rapid increase occurred at the beginning of storage. A similar, but more rapid increase occurred in apples held at 18°-20°C. after picking. In apples that had reached nearly maximum oil content at 1°C., removal to 18°-20°C. resulted in a decrease. Parallel changes occurred in the "total fatty acids."

The iodine number increased with increasing concentration of oil, indicating that the substances produced after picking are more unsaturated than those originally present. After prolonged storage the iodine number decreased.

In 1947 two pickings were made both at Orange, N.S.W., and Wantirna, Vic. The apples were stored at 0°C., and all determinations were made immediately after removal from this temperature. The results are given in Table 2.

TABLE 2
CHANGES IN OIL CONTENT IN 1947

District	Date of Picking	Weeks at 0°C.	Oil (mg./sq. cm.)	Iodine No. of Oil (Wijs)	Total Fatty Acids (μ E/sq. cm.)
Orange, N.S.W.	26.iii.47	0	0.04	58	—
		10	0.07	104	0.31
		20	0.18	—	—
		30	0.18	112	0.60
	16.iv.47	0	0.07	94	—
		10	0.16	131	0.47
		20	0.18	138	0.66
		30	0.21	136	0.73
Wantirna, Vic.	9.iv.47	0	0.05	63	0.21
		10	0.14	129	0.46
		20	0.16	138	0.53
		30	0.22	127	0.71
	1.v.47	0	0.10	86	0.42
		10	0.19	145	0.52
		20	0.22	124	0.72
		30	0.28	134	1.06

In all samples the oil increased at 0°C. to 3-4 times the original concentration. The later pickings had a higher oil content throughout the storage period. Similar changes occurred in the "total fatty acids." The iodine number of the oil increased markedly during the first 10 weeks and changed very little subsequently.

TABLE 3
CHANGES IN "TOTAL FATTY ACIDS" IN 1949
(Picked April 12, 1949 at Orange, N.S.W.)

Weeks at 0°C.	Concentration (μ E/sq. cm.) After Storage at 20°C. for			
	0 Weeks	2 Weeks	4 Weeks	6 Weeks
1	0.189	0.455	0.615	0.688
11	0.419	0.652	0.677	0.676
21	0.558	0.619	0.665	0.636
31	0.562	0.574	0.557	0.545

The data in Tables 1 and 2 indicate a significant correlation between oil and "total fatty acids" ($r = 0.931$, $P < 10^{-9}$). As the oil has a higher saponification number and increases much more than the wax during storage, most

of the increase of "total fatty acids" is due to increase of oil. The mean ratio of oil content (as mg./sq. cm.) to "total fatty acids" (as $\mu\text{E}/\text{sq. cm.}$) is 0.32.

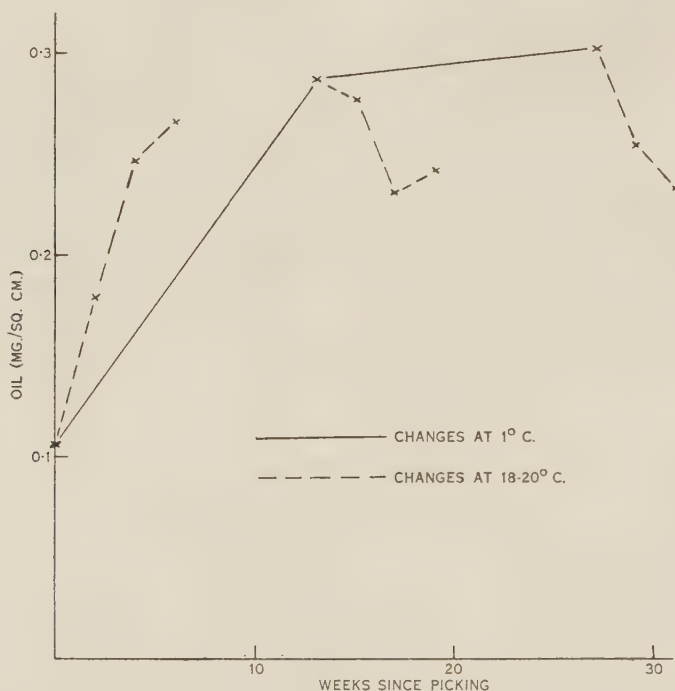


Fig. 1.—Changes in oil content in 1945 (picked April 10, 1945, at Orange, N.S.W.).

Further studies were made in 1949 with apples from Orange, N.S.W. The apples were stored at 0°C. and samples were removed to 20°C. at intervals. Determinations were made of "total fatty acids," which are an approximate index of the oil content. The results are given in Table 3 and Figure 2. "Total fatty acids" can be multiplied by 0.32 to give an approximate estimate of oil content.

Increases were obtained both at 0°C. and 20°C. similar to those obtained in 1945. The oil content appeared to reach a somewhat higher level at 20°C. Samples removed to 20°C. after 31 weeks at 0°C. did not change significantly, although the "total fatty acids" were still below the level attained previously at 20°C.

IV. INCREASE IN ACIDS AND UNSAPONIFIABLE MATTER OF OIL

The increase of oil during storage indicates a definite production of oily substances by the apple tissue. These substances may be produced by the epidermal cells and secreted into the cuticle; or they may be transported to the surface from other parts of the apple.

The production of oil was studied in more detail in 1950. The apples were picked at Orange on April 26. Samples were analysed immediately after picking and also after 6 weeks at 20°C. The oil was separated from the wax and weighed. It was then saponified, and the acids and unsaponifiable matter were separated and weighed. The acid fraction was separated with boiling light

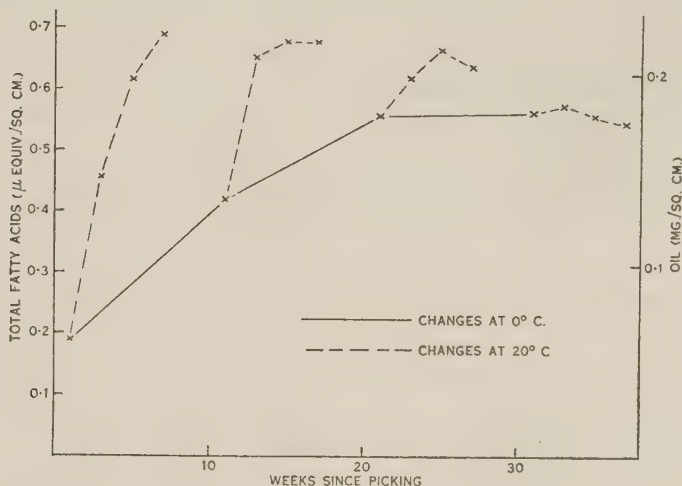


Fig. 2.—Changes in "total fatty acids" in 1949 (picked April 12, 1949, at Orange, N.S.W.).

petroleum (about 40 ml. per g.) into soluble and insoluble portions. Acid and hydroxyl numbers were determined on the acid fractions. Iodine numbers were determined by the Wijs method on the soluble acids and the unsaponifiable matter. The results are given in Table 4.

TABLE 4
CHANGES IN OIL SUBSTANCES AT 20°C.

Determination	Immediately after Picking	After 6 Weeks at 20°C.
Original oil (mg./sq. cm.)	0.099	0.226
Soluble acids (mg./sq. cm.)	0.062	0.135
Soluble acids (acid no.)	158	162
Soluble acids (hydroxyl no.)	64	39
Soluble acids (iodine no.)	100	102
Insoluble acids (mg./sq. cm.)	0.009	0.012
Insoluble acids (acid no.)	88	58
Insoluble acids (hydroxyl no.)	372	348
Unsaponifiable matter (mg./sq. cm.)	0.020	0.061
Unsaponifiable matter (iodine no.)	84	103

The increase in oil involved considerable increases both in acids and unsaponifiable matter. The iodine number of the soluble acids hardly changed

during storage but the iodine number of the unsaponifiable matter increased considerably. The increase in iodine number of the oil appeared to be due mainly to increases in the iodine number and proportion of unsaponifiable matter.

The dark insoluble acids appeared to be mainly complex polyhydroxy acids. They may be products of oxidation of unsaturated acids. With a volume of light petroleum equivalent to 40 ml. per g. some hydroxy acids passed into the soluble portion, which had an appreciable hydroxyl number. However, the data are sufficient to show that the acids produced during storage are predominantly non-hydroxy. The increase of titratable acid in the soluble portion was $0.215 \mu\text{E/sq. cm.}$, while the increase of hydroxyl was only $0.023 \mu\text{E/sq. cm.}$ Hence there would not be more than one mole of hydroxy acid produced for every 10 moles of soluble acid.

V. RELATION BETWEEN OIL CONTENT AND RESISTANCE OF SKIN TO GASEOUS DIFFUSION

During 1947, measurements were made of the resistance of the skin to diffusion of oxygen in parallel with the determinations of oil content. The measurements were made at 20°C. immediately after removal from 0°C. The results are given in Table 5.

TABLE 5
CHANGE IN RESISTANCE OF SKIN AT 0°C.

District and Picking	Weeks at 0°C.	Oil (mg./sq. cm.)	Resistance of Skin	
			Individual Apples	Mean
Orange, N.S.W. 26.iii.47	0	0.04	0.4, 0.6, 0.6, 0.8, 0.8, 0.8, 0.9, 0.9, 1.1, 1.3, 2.1, 2.5	1.1
	10	0.07	0.8, 0.9, 0.9, 1.1, 1.3	1.0
	20	0.18	1.0, 1.5, 1.6, 1.9, 2.2, 2.3	1.7
	30	0.18	—	—
Orange, N.S.W. 16.iv.47	0	0.07	1.2, 1.4, 1.5, 3.5	1.9
	10	0.16	1.3, 1.4, 1.5, 2.1, 2.7	1.8
	20	0.18	1.6, 1.6, 2.1, 3.2, 3.4, 5.4	2.9
	30	0.21	4.3, 4.6, 5.7, 5.9, 6.4, 6.5	5.6
Wantirna, Vic. 9.iv.47	0	0.05	1.3, 1.3, 1.7, 1.7, 1.8, 2.6	1.7
	10	0.14	1.6, 1.8, 2.1, 2.3, 2.5	2.1
	20	0.16	1.8, 2.3, 2.3, 2.7, 3.5, 4.8	2.9
	30	0.22	1.0, 1.4	1.2
Wantirna, Vic. 1.v.47	0	0.10	0.8, 1.9, 2.1, 2.8, 3.1	2.1
	10	0.19	1.0, 1.1, 1.6, 1.9, 2.0	1.5
	20	0.22	2.0, 2.2, 2.3, 2.8, 2.9, 3.2	2.6
	30	0.28	1.3, 2.1, 2.9	2.1

Measurements of resistance were usually made on 5-6 individual apples, but some of the apples in the last removal were lost through mould. In

general, the resistance did not change significantly in the first 10 weeks but increased definitely in the second 10 weeks. In the last 10 weeks the resistance of the second picking from Orange continued to increase. The resistance of the apples from Wantirna appeared to decrease in the last 10 weeks, but the measurements were rather few. Similar trends were observed by Hackney (1943) who found that the resistance increased during several months at 1°C. and sometimes decreased after prolonged storage.

There was no definite correlation between resistance of skin and concentration of oil. The oil generally increased continuously during storage. The resistance of the skin is not determined solely by the concentration of oil but probably depends on other factors such as the distribution and physical state of the oil and other components of the natural coating.

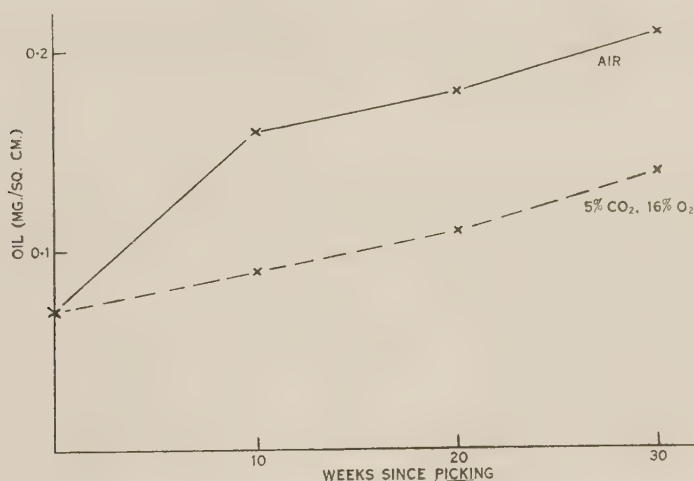


Fig. 3.—Changes in oil content at 0°C. in air and 5 per cent. CO₂, 16 per cent. O₂ (picked April 16, 1947, at Orange, N.S.W.).

VI. EFFECT OF "GAS" STORAGE ON INCREASE OF OIL CONTENT

Apples were picked at Orange on April 16, 1947 (second picking) and stored at 0°C. Samples were stored both in air and in an atmosphere containing 5 per cent. of carbon dioxide and 16 per cent. of oxygen and obtained by controlled ventilation. Storage in atmospheres containing more carbon dioxide and less oxygen than ordinary air is known as "gas" storage. Determinations of oil content were made on samples stored in air and in "gas." The results are given in Table 6 and Figure 3.

The increase in oil was definitely reduced by "gas" storage. It has been shown that other ripening changes, e.g. colouring, softening, hydrolysis of polysaccharides, and loss of acid are also retarded by "gas" storage.

VII. CHANGES IN WAX FRACTION DURING STORAGE

The results for 1945 are given in Table 7 and the results for 1947 and 1948 in Table 8.

TABLE 6
CHANGES IN OIL CONTENT IN AIR AND "GAS" STORAGE

Weeks at 0°C.	Oil (mg./sq. cm.) in	
	Air	5% CO ₂ , 16% O ₂
0	0.07	0.07
10	0.16	0.09
20	0.18	0.11
30	0.21	0.14

Increases during cold storage varied from negligible to about 40 per cent. of the initial concentration. The average increase was about 25 per cent. Most of this increase occurred in the last few weeks of storage.

TABLE 7
CHANGES IN WAX FRACTION IN 1945
(Picked April 10, 1945 at Orange, N.S.W.)

Weeks at 1°C.	Concentration (mg./sq. cm.) After Storage at 18°-20°C. for			
	0 Weeks	2 Weeks	4 Weeks	6 Weeks
0	0.253	0.258	0.250	0.220
13	0.232	0.244	0.242	0.265
27	0.282	0.286	0.264	—

TABLE 8
CHANGES IN WAX FRACTION IN 1947 AND 1948

District	Date of Picking	Concentration (mg./sq. cm.) After Storage at 0°C. for			
		0 Weeks	10 Weeks	20 Weeks	30 Weeks
Orange,	26.iii.47	0.23	0.28	0.25	0.26
N.S.W.	16.iv.47	0.24	0.26	0.33	0.29
	5.v.48	0.199	0.208	0.209	0.217
Wantirna,	9.iv.47	0.28	0.31	0.26	0.37
Vic.	1.v.47	0.31	0.29	0.33	0.44

VIII. CHANGES IN URSOLIC ACID FRACTION DURING STORAGE

The changes during cold storage are given in Table 9. Increases up to 40 per cent. were also obtained in the ursolic acid fraction. Most of the increase usually occurred in the last 10 weeks at 0°C.

IX. CHANGES IN "CUTIN" FRACTION DURING STORAGE

The changes in the acids of the "cutin" fraction are given in Table 10. A significant increase of about 20 per cent. occurred in the last 10 weeks at 0°C., but no other differences were significant. It seems that significant increases in the wax, ursolic acid, and "cutin" fractions usually appear after about 20 weeks at 0°C.

TABLE 9
CHANGES IN URSOLIC ACID FRACTION IN 1947 AND 1948

District	Date of Picking	Concentration (mg./sq. cm.) After Storage at 0°C. for			
		0 Weeks	10 Weeks	20 Weeks	30 Weeks
Orange,	26.iii.47	0.33	0.40	0.36	0.46
N.S.W.	16.iv.47	0.37	0.44	0.43	0.54
	5.v.48	0.373	0.367	0.439	0.425
Wantirna,	9.iv.47	0.44	0.50	0.42	0.60
Vic.	1.v.47	0.44	0.48	0.46	0.48

X. DISCUSSION

The increase of oil during storage can be considered in relation to the lipid metabolism of the apple. Evidence has been obtained that the apple cells produce fatty acids (mostly esterified) and other fatty substances. The production of fatty substances is only a small component of the total metabolism (although it may be a major component in certain cells), but its influence on other metabolic processes is probably quite considerable. The accumulation of oil may affect the resistance of the skin to diffusion of oxygen and hence the concentration of internal oxygen. The latter affect respiration and ripening changes. Changes in the resistance of the skin to water loss may also have effects on metabolism.

TABLE 10
CHANGES IN ACIDS OF "CUTIN" FRACTION IN 1949
(Picked April 12, 1949 at Orange, N.S.W.)

Weeks at 0°C.	Concentration (mg./sq. cm.) After Storage at 20°C. for			
	0 Weeks	2 Weeks	4 Weeks	6 Weeks
1	0.398	0.392	0.378	0.413
11	0.368	0.389	0.370	0.437
21	0.401	0.411	0.392	0.413
31	0.474	0.458	0.449	0.432

The production of oil was generally most rapid at the beginning of the storage period and then declined. In many cases the concentration of oil finally approached a maximum value. The apparent cessation of oil production may be due to (a) inactivation of the lipid-producing systems, (b)

exhaustion of lipid precursors, or (c) the effect of increasing oil concentration on the lipid metabolism. The first alternative is quite plausible, as there is a failure of other synthetic mechanisms during senescence.

The identification of the fatty acids of the oil is still being attempted, and preliminary results indicate a variation of chain length from 12 to 22 carbon atoms. The volatile esters of Granny Smith apples have been shown to be derived from lower acids and alcohols with a maximum chain length of six carbon atoms (Thompson 1951), and their production has recently been studied by Thompson and Huelin (1951). In 1949, determinations of volatile ester production and of the "total fatty acids" of the natural coating were made on corresponding samples from the same source. The production of volatile esters (Thompson and Huelin 1951) can be linked with the accumulation of non-volatile fatty acids shown in Table 3 and Figure 2. The production of volatile esters reached its maximum rate much later than the accumulation of the non-volatile fatty acids. This is demonstrated in Table 11, where the total increase of each group at 20°C. is given for fortnightly periods.

TABLE 11
TOTAL INCREASE OF VOLATILE ESTERS AND NON-VOLATILE ACIDS AT 20°C.

Weeks at 0°C.	Days at 20°C.	Volatile Esters (mM/kg.)	Non-Volatile Acids (mE/kg.)
1	0-14	0.04	0.28
	14-28	0.32	0.17
	28-42	0.90	0.08
11	0-14	0.15	0.24
	14-28	0.21	0.03
	28-42	2.52	0
21	0-14	1.09	0.06
	14-28	1.23	0.05
	28-42	0.06	-0.03
31	0-14	0.80	0.01
	14-28	0.74	-0.02
	28-42	0.60	-0.01

In the first series of measurements at 20°C., an increasing rate of volatile ester production was accompanied by a declining rate of non-volatile acid production. A similar result was obtained after 11 weeks at 0°C. In later removals, volatile ester production was comparatively high initially, while non-volatile acid production had already fallen to a low value. Thompson and Huelin (1951) have obtained evidence that the increasing rate of volatile ester production is associated with an accumulation of volatile alcohols.

The complex relation of oil production to other ripening changes is emphasized by the effect of "gas storage." This technique involves a decrease of oxygen and an increase of carbon dioxide concentration in the external atmosphere. The slower ripening which occurs in "gas storage" is probably due to similar changes in the internal atmosphere. By reducing oil accumulation "gas storage" could delay the increase of skin resistance and hence modify its more direct effect upon the internal atmosphere.

XI. ACKNOWLEDGMENTS

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XII. REFERENCES

- HACKNEY, F. M. V. (1943).—*Proc. Linn. Soc. N.S.W.* 68: 33.
HUELIN, F. E., and GALLOP, R. A. (1951).—*Aust. J. Sci. Res. B* 4: 526.
THOMPSON, ADRIENNE R. (1951).—*Aust. J. Sci. Res. B* 4: 283.
THOMPSON, ADRIENNE R., and HUELIN, F. E. (1951).—*Aust. J. Sci. Res. B* 4: 544.
TROUT, S. A., HALL, E. G., ROBERTSON, R. N., HACKNEY, F. M. V., and SYKES, S. M. (1942).—*Aust. J. Exp. Biol. Med. Sci.* 20: 219.

VOLATILE PRODUCTS OF APPLES

II. PRODUCTION OF VOLATILE ESTERS BY GRANNY SMITH APPLES

By ADRIENNE R. THOMPSON* and F. E. HUELIN*

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Summary

Granny Smith apples were stored at 0°C., and samples were removed at intervals for determination of volatile ester production at 20°C.

In early removals ester production at 20°C. increased to a maximum and then decreased. In later removals the increase was much less and finally became negligible. Ester production at 0°C. appeared to increase steadily.

Early picking reduced ester production and a higher rate of air flow increased it. Reducing the oxygen concentration to 6 per cent. first increased and then decreased ester production in comparison with air.

The metabolic significance of the results is discussed.

I. INTRODUCTION

Evidence has been presented that superficial scald, a functional disorder of cold-stored apples, may be caused by the volatile substances produced by the fruit itself. In view of the possible importance of esters in relation to scald, as indicated by the experiments of Brooks, Cooley, and Fisher (1919), it was decided to measure the production of volatile esters. The ester fraction has not previously been determined separately, although other workers have determined the production of "odorous" volatiles after absorption in concentrated sulphuric acid. The absorbed volatiles were determined by wet oxidation with chromic acid and measurement of the carbon dioxide produced (Potter and Griffiths 1947; Griffiths and Potter 1949). This method, however, is purely empirical.

The identification of the acids and alcohols, free and combined as esters, given off by Granny Smith apples has been described in an earlier paper (Thompson 1951).

The measurements of volatile ester production have all been made with Granny Smith apples. The first measurements were made in 1949 on a series of samples removed to 20°C. after various periods at 0°C. Definite trends in ester production were observed, and further measurements were made in 1950, when the effect of reducing the oxygen concentration was investigated. Some determinations of volatile acids and alcohols in the fruit were also made in 1950.

II. EXPERIMENTAL TECHNIQUE

(a) Collection of Esters

Air, purified by combustion of organic matter and absorption of carbon dioxide, was passed over 5-10 kg. of Granny Smith apples held at 20°C.

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either in a series of four desiccators or in a galvanized iron drum. The rate of flow varied from 10 to 40 l./hr. in different experiments. The volatile substances were absorbed by passing the air first through a U-tube cooled in an ice-calcium chloride mixture and then through two spiral absorbers, each containing 30 ml. of ether and cooled in a solid carbon dioxide-alcohol mixture. After 6-24 hr. the absorbers were disconnected, emptied into a separating funnel and washed out with ether. The aqueous layer was then separated off and extracted with ether. The combined ether extracts were dried over calcium chloride and made up to volume (usually 100 ml.) in a volumetric flask.

Initially the contents of the second absorber were made up and analysed separately. It was found that, with a flow rate of 10 l./hr., an average of 95 per cent. of the esters was collected in the U-tube and first spiral absorber. Although little ester was collected in the U-tube, some water condensed which would otherwise have blocked the spiral absorber.

(b) Determination of Esters

The apple esters were determined colorimetrically in 5 ml. of the ether solution by the procedure described previously (Thompson 1950). This method involves conversion of the esters to hydroxamic acids, which are determined as the coloured ferric hydroxamate complexes.

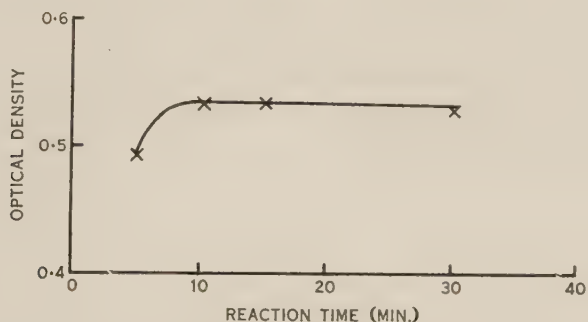


Fig. 1.—Formation of hydroxamic acids from apple esters.

The method is applicable to the mixed apple esters, since tests have shown that the reaction with hydroxylamine is complete in 15 min. (Fig. 1) and Beer's Law is obeyed in the same way as for pure esters. Other tests have shown that the procedure of drying with calcium chloride, together with other manipulations, does not result in a loss of more than 3 per cent. Acetaldehyde does not interfere if its concentration in the ether solution does not exceed 0.1M.

Since *n*-butyrate appeared to predominate in the apple esters during 1949 (Thompson 1951), the apple ester concentration was calculated by assuming a ratio of 2.12×10^{-3} M per unit optical density, as previously found for ethyl butyrate (Thompson 1950). This ratio does not differ appreciably from that for acetate or propionate.

(c) Determination of Volatile Acids and Alcohols in Fruit

Cut sections of whole apples (approx. 2 kg.) were steam-distilled for 3 hr. through a double surface condenser into a receiver cooled in ice. The receiver was connected to a second trap containing water cooled in ice. The distillate was mixed with the water from the trap and divided into two equal portions.

Total volatile acids (free and esterified) were determined in one portion. Sodium hydroxide was added to a concentration of approximately 0.1N, and the solution was allowed to stand overnight. The solution was then acidified with sulphuric acid and re-distilled. After concentrating to approximately 100 ml., a further 100 ml. of water was added and distillation continued to the same final volume. Nitrogen was bubbled through the distillate (to remove carbon dioxide) and the acid determined by titration with 0.1N sodium hydroxide, using a mixture of neutral red and methylene blue as indicator.

Total volatile alcohols were determined by the method of Fidler (1934). The second portion of the first distillate was oxidized with chromic acid and re-distilled. The figure obtained by titration of the final distillate included acid originally present as well as that produced by oxidation of alcohol. By subtracting the figure for volatile acids, the figure for volatile alcohols was obtained.

(d) Respiration and Composition of Internal Atmosphere

The respiration (CO_2 production) of approximately 5 kg. lots of apples was determined by the Pettenkoffer method. The concentration of oxygen and carbon dioxide in the internal atmosphere of individual apples was determined by the method of Trout *et al.* (1942).

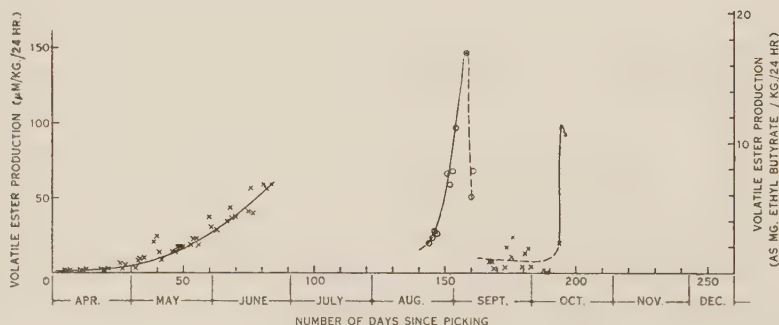


Fig. 2.—Production of volatile esters by Granny Smith apples at 20°C. after removal from storage at 0°C. (picked March 30, 1949).

III. VOLATILE ESTER PRODUCTION IN AIR

Two pickings of Granny Smith apples were made at Orange, N.S.W., on March 30 and April 12, 1949. The first picking was made a few days before commercial picking and the second was commercially mature. Apples of both

pickings were stored at 0°C. and samples were removed at intervals for determination of volatile ester production at 20°C. Apples of the first picking were removed after 0, 20, and 23 weeks and apples of the second picking after 1, 6, 11, 16, 22, 26, and 31 weeks. Measurements were continued for as long as the fruit was sound and free from rots. The values of volatile ester production for the first picking are given in Figure 2 and for the second picking in Figure 3. In these experiments measurements were made with 10 kg of fruit and a rate of air flow of 10 l./hr. There was frequently considerable fluctuation in individual readings, but curves of best fit were drawn.

Volatile ester production shows a similar trend in both pickings, although the data for the second picking are more complete. The ester production of the first picking was generally lower than that of the second picking. In the first removal the ester production increased steadily from a very low value over a period of about 10 weeks. In subsequent removals an initial delay was followed by a rapid rise to a maximum and a fairly rapid fall. In later removals the rise became much less and finally negligible.

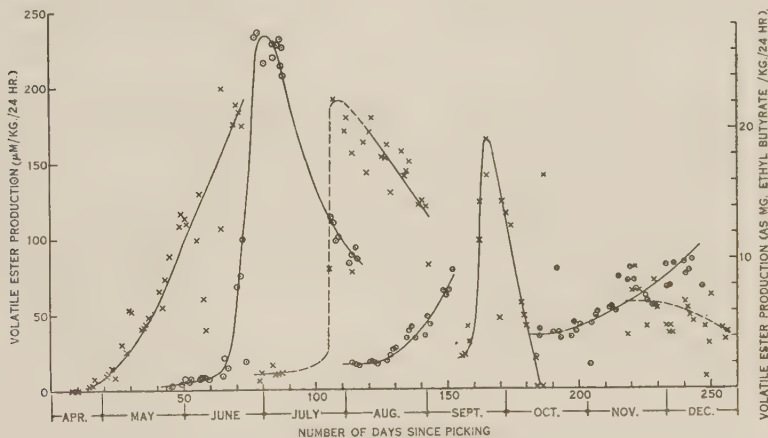


Fig. 3.—Production of volatile esters by Granny Smith apples at 20°C. after removal from storage at 0°C. (picked April 12, 1949).

Determination of carbon dioxide in the air leaving the desiccators gave a concentration of about 2 per cent. It has been shown by Potter and Griffiths (1947) and Griffiths and Potter (1949) that accumulation of carbon dioxide or organic volatiles can reduce the volatile ester production. For comparison with the sample of the second picking removed after 26 weeks, an additional sample was removed after 27 weeks. The ester productions were measured with rates of air flow of 10 and 40 l./hr., and the results are given in Figure 4. Increasing the rate of flow to 40 l./hr. approximately doubled the ester production over a period of 7 weeks. Subsequently the production at the higher rate of air flow appeared to decrease earlier.

The effect of accumulated carbon dioxide or organic volatiles associated with the lower rate of air flow must be considered as a possible factor in interpreting the results of Figures 2 and 3. However, as the higher rate of air flow increased the volatile ester production in approximately constant ratio over several weeks, it is probable that the general form of the curves has not been significantly affected.

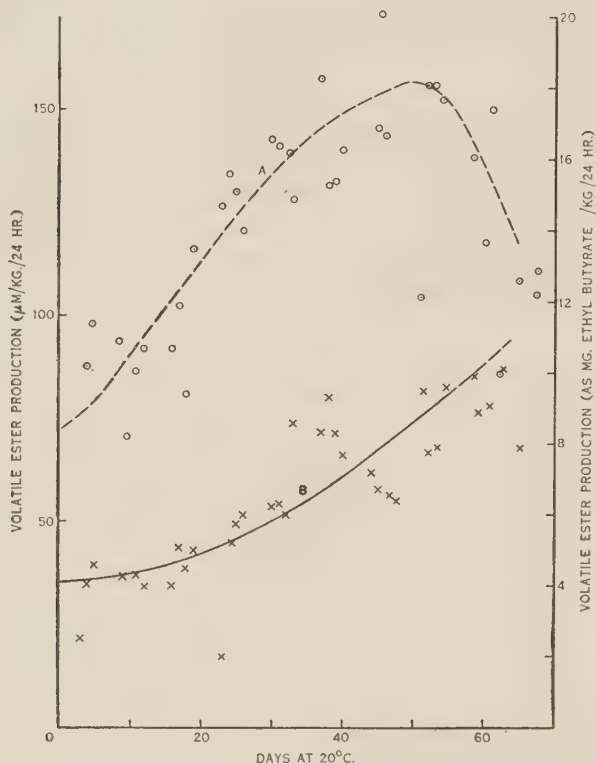


Fig. 4.—Effect of flow rate on production of volatile esters by apples.

A, flow rate 40 l./hr. Apples stored 27 weeks at 0°C.

B, flow rate 10 l./hr. Apples stored 26 weeks at 0°C.

Measurements of respiration were made on apples of the second picking removed to 20°C. after 6, 11, 16, and 22 weeks at 0°C. The results are given in Figure 5. The curves of respiration and ester production followed quite different trends. Respiration fell rapidly at first and then more slowly. In the first three curves the end of the rapid fall in respiration coincided approximately with the beginning of the rapid rise in ester production.

The measurements of volatile ester production were all made at 20°C., as the production at 0°C. is very low. However, the trend of ester production at 0°C. can be determined by assuming a constant temperature coefficient, i.e. a constant ratio between initial ester production at 20°C. and the corresponding

production at 0°C. The values of initial ester production at 20°C. in the second picking are plotted in Figure 6. The curve obtained shows a steady rise, which suggests a continuous increase in ester production at 0°C.

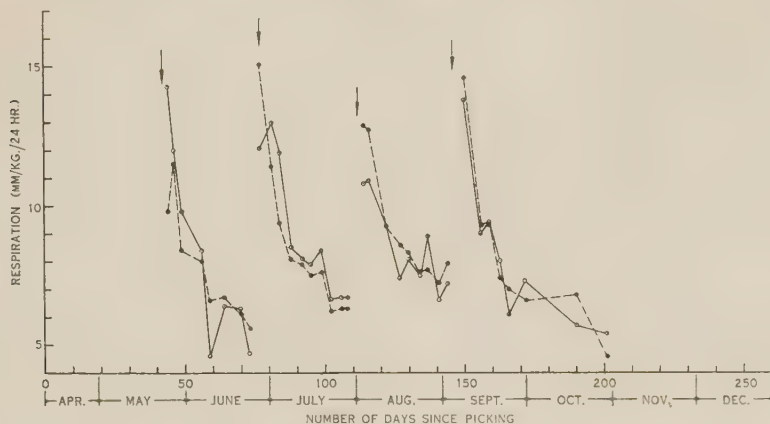


Fig. 5.—Respiration of Granny Smith apples at 20°C. after removal from storage at 0°C. (picked April 12, 1949). Arrows indicate the day on which the apples were removed from 0°C. to 20°C. Curves show duplicate estimations.

The changes in volatile ester production are difficult to interpret without further data. In view of the possibility of a relation between ester production and internal oxygen concentration, which has been shown to decrease with length of storage (Hackney 1943), it was decided to lower the concentration of oxygen in the external atmosphere. The concentration of alcohol, which may be a factor in ester production, usually increases if the concentration of oxygen is reduced sufficiently.

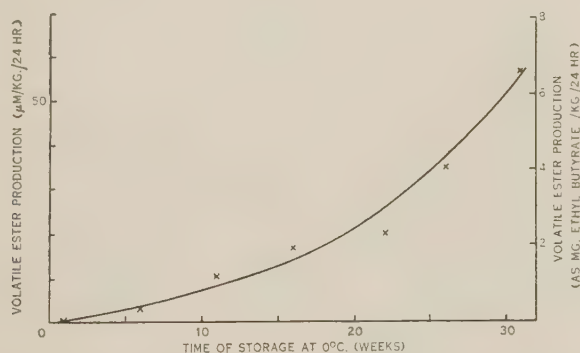


Fig. 6.—Production of volatile esters immediately after removal from storage at 0°C.

IV. EFFECT OF REDUCED OXYGEN CONCENTRATION

Granny Smith apples were picked at Orange on April 19, 1950, and stored at 0°C. After 3 and 8 weeks samples were removed to 20°C. for measure-

ments of volatile ester production. One 5 kg. sample of each removal was held in air and the other in an atmosphere containing 6 per cent. oxygen and 94 per cent. nitrogen. The rate of gas flow was 20 l./hr. The results are given in Figure 7.

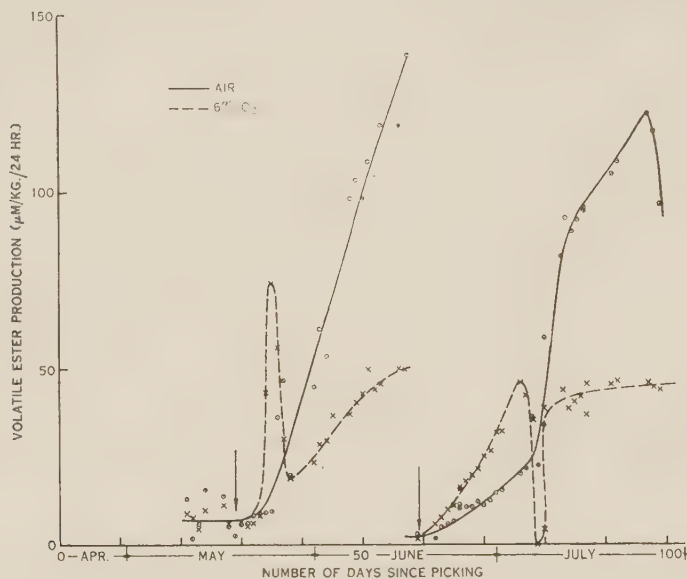


Fig. 7.—Production of volatile esters in air and 6 per cent. oxygen at 20°C. after removal from 0°C. (picked April 19, 1950). Arrows indicate the change from air to 6 per cent. oxygen.

The curves of ester production in air were similar to those obtained in 1949. After an initial delay, ester production increased rapidly. The measurements on the second removal were continued long enough to reach the maximum. Ester production in 6 per cent. oxygen increased rapidly at first to a maximum well above the production in air at that time. It then fell to a minimum and finally increased again. In the final stage the rate of ester production in 6 per cent. oxygen was well below the rate in air.

Measurements of respiration and the composition of the internal atmosphere were made on corresponding samples and the results are given in Figure 8. Reducing the external oxygen reduced the internal oxygen to a similar concentration but had little effect on the internal carbon dioxide or respiration. The respiration tended to fall but not nearly as rapidly as in the previous year.

V. VOLATILE ACIDS AND ALCOHOLS

On completing the measurements of volatile ester production in air and 6 per cent. oxygen, the samples were used for determination of volatile acids and alcohols in the whole fruit. In addition, data were obtained on samples which

had been stored at 0°C. for 10 weeks, and these were regarded as representing the approximate level of acids and alcohols before removal to 20°C. The results are given in Table 1.

TABLE 1
VOLATILE ACIDS AND ALCOHOLS IN GRANNY SMITH APPLES

Days at 20°C.	Acid (mE/kg.)		Alcohol (mE/kg.)	
	Air	6% O ₂	Air	6% O ₂
0	0.33	—	0.20	—
38 (1st removal)	0.72	0.20	1.59	0.64
44 (2nd removal)	0.11	0.13	2.22	0.52

The most important result is that the alcohol content of the fruit increased at 20°C. as well as the ester production. At the end of each experiment the alcohol content in 6 per cent. oxygen was below that in air and corresponded to a lower rate of ester production. This result will be discussed more fully in the next section.

Volatile acid content showed some increase in the first removal in air, but not in the other samples.

VI. DISCUSSION

The drift in volatile ester production in air can be briefly summarized as follows. At 0°C. ester production increased steadily for several months. In samples removed to 20°C. ester production rose to a maximum and then fell. In later removals the rise became less and finally negligible. The increase in ester production appeared to be associated with a greater production and accumulation of alcohols by the fruit.

Production of volatile alcohols can probably be regarded as due to comparatively slow side reactions of the normal aerobic respiration. One would expect the rates of such reactions to depend on the concentration of reduced diphosphopyridine nucleotide (DPNH₂) and other reducing agents. Pyruvic acid, an important intermediate of respiration, can be converted to ethanol by decarboxylation to acetaldehyde and subsequent reduction. The reduction of acetaldehyde is effected by DPNH₂, which results from the reduction of DPN by respiratory substrates such as 3-phosphoglyceraldehyde. One can postulate the formation of *n*-propanol by reduction of pyruvic acid and the formation of longer-chain alcohols by linkage of shorter fragments. Primary alcohols up to caproic have been identified as products of apples (Thompson 1951; Walls 1942; White 1950).

Fidler (1933) has shown a continuous increase in the alcohol content of apples during storage, and attributes it to a gradual replacement of normal oxidative respiration by "zymasis" or fermentation. This and other changes associated with ripening and subsequent deterioration are known collectively as senescence. In oxidative respiration DPNH₂ is oxidized through a series of linked enzyme systems, the last of which uses atmospheric oxygen. A decline

in activity of these oxidative systems (due to decreasing efficiency or lowering of internal oxygen concentration after prolonged storage in air) would increase the concentration of DPNH_2 and lead to increased alcohol production. This is the most probable explanation for the increase of alcohol content in air.

The changes in ester production obtained in 1949 (Fig. 3) can now be considered further. The initial increases in ester production could be due to an increasing concentration of alcohol, but the subsequent fall and the lack of increase after prolonged storage must have another explanation. The available evidence indicates a continuous rise in alcohol content during storage, hence the fall in ester production could be due to a decline in volatile acid production or subsequent esterification.

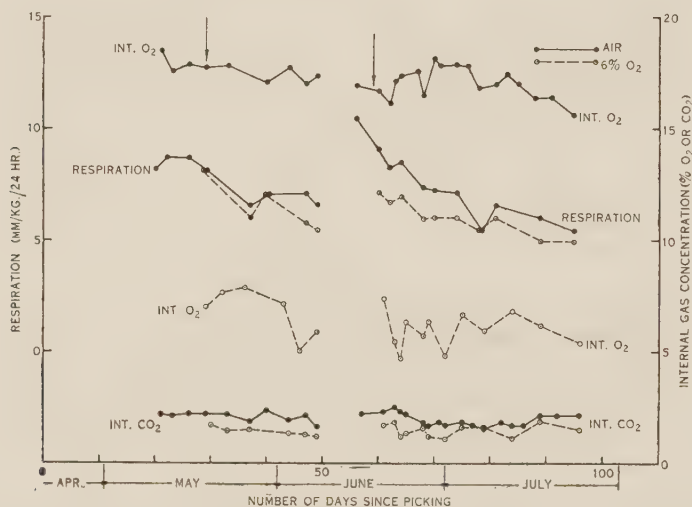


Fig. 8.—Respiration and internal gas concentration in air and 6 per cent. oxygen at 20°C. after removal from 0°C. (picked April 19, 1950). Arrows indicate the change from air to 6 per cent. oxygen.

The effect of reducing the oxygen concentration from 21 to 6 per cent. (Fig. 7) is difficult to interpret. The ester production in 6 per cent. oxygen can be considered in three stages: rise to maximum (1), fall to minimum (2), and subsequent rise (3). Reducing the oxygen might be expected to reduce oxidative metabolism and increase the production of alcohol and esters. This appeared to be the first result of the low oxygen (stage 1), but subsequently an adjustment (stage 2) occurred to nullify this effect.

In stage 3, alcohol content and ester production in 6 per cent. oxygen were less than in air. Ester production increased in 6 per cent. oxygen but much less rapidly than in air. The effect of reduced oxygen at this stage is possibly not a direct effect on oxidative metabolism but a more general effect in delaying senescence. Senescence involves, among other changes, a gradual decline in the efficiency of oxidative systems and an increase of alcohol production.

Reduction of oxygen concentration to 6 per cent. could arrest this decline, at least in the early stages of senescence. However, after prolonged storage the oxidative systems may be unable to function adequately in 6 per cent. oxygen.

As previously mentioned, the ester production of the first picking in 1949 was generally lower than that of the second picking. As earlier pickings are more liable to superficial scald, this result does not support the theory that volatile esters are directly concerned in this disorder. This theory is now somewhat doubtful, as experiments with synthetic esters in this laboratory have failed to provide evidence in its support.

VII. ACKNOWLEDGMENTS

The authors wish to thank Dr. R. N. Robertson for many helpful discussions and suggestions; Mr. T. J. Riley for determinations of respiration and composition of internal atmospheres; and Miss H. Smith and Mr. B. Kennett for technical assistance.

VIII. REFERENCES

- BROOKS, C., COOLEY, J. S., and FISHER, D. F. (1919).—Nature and control of apple scald. *J. Agric. Res.* **18**: 211-40.
- FIDLER, J. C. (1933).—Studies in zymasis. IV. The accumulation of zymasic products in apples during senescence. *Biochem. J.* **27**: 1614-21.
- FIDLER, J. C. (1934).—Studies in zymasis. VII. The estimation of the ethyl alcohol and acetaldehyde content of apples. *Biochem. J.* **28**: 1107-20.
- GRIFFITHS, D. G., and POTTER, N. A. (1949).—Effects of the accumulation of volatile substances produced by apples in gas storage. *J. Pomol.* **25**: 10-8.
- HACKNEY, F. M. V. (1943).—Studies in the metabolism of apples. II. The respiratory metabolism of Granny Smith apples of commercial maturity after various periods of cool storage. *Proc. Linn. Soc. N.S.W.* **68**: 33-47.
- POTTER, N. A., and GRIFFITHS, D. G. (1947).—The effects of temperature and gas mixtures on the production of volatile substances by apples during storage. *J. Pomol.* **23**: 171-7.
- THOMPSON, ADRIENNE R. (1950).—A colorimetric method for the determination of esters. *Aust. J. Sci. Res. A* **3**: 128-35.
- THOMPSON, ADRIENNE, R. (1951).—Volatile products of apples. I. Identification of acids and alcohols. *Aust. J. Sci. Res. B* **4**: 283.
- TROUT, S. A., HALL, E. G., ROBERTSON, R. N., HACKNEY, F. M. V., and SYKES, S. M. (1942).—Studies in the metabolism of apples. I. Preliminary investigations on internal gas composition and its relation to changes in stored Granny Smith apples. *Aust. J. Exp. Biol. Med. Sci.* **20**: 219-31.
- WALLS, L. P. (1942).—The nature of the volatile products from apples. *J. Pomol.* **20**: 59-67.
- WHITE, J. W. (1950).—Composition of a volatile fraction of apples. *Food Res.* **15**: 68-78.

A COMPETITIVE INHIBITOR OF TYROSINASE

By C. WARNER*

[Manuscript received July 2, 1951]

Summary

The kinetics of the activation of catechol by tyrosinase prepared from the potato and the mushroom, and of its inhibition by sodium *m*-hydroxybenzoate, have been studied. The enzyme-substrate dissociation constants differed markedly between the two enzyme sources (K_s potato = 5.0mM, K_s mushroom = 0.28mM), as did also the enzyme-inhibitor dissociation constants (K_i potato = 2.5mM, K_i mushroom = 0.6mM). For both enzyme preparations sodium *m*-hydroxybenzoate met the requirements of a competitive inhibitor.

I. INTRODUCTION

Although there has been a considerable amount of work done on the enzyme tyrosinase, it has been mainly concerned with the distribution of the enzyme in plant or animal species (see e.g. Onslow 1931) or, more frequently, with the mechanism of the action of the enzyme on monohydric and dihydric phenols (reviewed in Nelson and Dawson 1944). A number of workers in the field have noticed inhibition of the enzyme by various substances, mainly those reacting with copper, but few have tried to identify the type of inhibition.

A search of the literature has revealed only one paper that examined the kinetics of tyrosinase according to the theory of Michaelis and Menten (1913) and its inhibition by a competitive inhibitor. This is a paper by Hackney (1948) on the tyrosinase of apples, and it is unfortunate that misconceptions of the criteria of competitive inhibition and some apparent mathematical errors should have marred the work; these will be discussed later.

This paper records a study of a competitive inhibitor of tyrosinase on more valid grounds. A preliminary examination of a number of possible aromatic inhibitors led to the selection of *meta*-hydroxybenzoic acid as one which inhibits the enzyme strongly and is apparently free of side effects; resorcinol did not appear to be satisfactory in the latter respect.

II. MATERIALS AND METHODS

Substrate.—Catechol, B.D.H. laboratory reagent "Pyrocatechol" was used.

Inhibitor.—Sodium *m*-hydroxybenzoate was prepared by neutralizing solid *m*-hydroxybenzoic acid (B.D.H. laboratory reagent) with the calculated amount of 10 per cent. NaOH and evaporating and allowing to crystallize. The pH of a solution of this salt was about 7.2.

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Enzyme.—Studies were commenced with a preparation from the common potato, *Solanum tuberosum*. Frozen potatoes were peeled, sliced, and blended in a Waring Blendor with enough 1M phosphate buffer of pH 7.5 to make the final concentration 0.001M. The mix was then filtered through Whatman No. 4 filter paper into *n*-butanol, keeping the whole system as cool as possible. The flocculent precipitate was filtered off and the excess butanol removed. A clear, brownish solution, stable in the refrigerator for several months if protected from air, resulted. No attempt was made to purify or concentrate this solution any further. The activity of the solution, measured according to the method of Miller *et al.* (1944) was about 20-40 units per ml. depending on the batch. For this investigation, several batches were pooled.

Later work was done with a commercial preparation of purified tyrosinase from the mushroom, *Psalliota campestris*, produced by the Syn-Zyme Laboratories of New York. This solution had an original activity of 3700 units per ml., and was diluted for use to about 30 units per ml.

Measurement of activity.—The common methods of measuring tyrosinase activity were all developed to study the concentration or activity of various enzyme preparations, not the effect of varying the concentration of substrate. The actual substrate concentrations used by the various authors were either completely arbitrary or else adjusted to that concentration which would give maximum activity, the "optimum concentration" of Nelson and co-workers. This is in effect the concentration of substrate at which the normal Michaelis curve shows no further appreciable rise. In all cases the methods as published demanded concentrations of substrate above those desirable for kinetic work. As a consequence, several different methods were studied, using low concentrations of substrate.

The methods examined were:

(1) *Manometric Method.*—Warburg manometers and vessels were used, with a reaction mixture of enzyme, substrate, and 0.07M phosphate buffer of pH 7.4 to a total volume of 3.0 ml. The temperature was maintained at 25°C. In some experiments 0.1 per cent. gelatin (Nelson and Dawson 1944) or *o*-phenylene diamine in equimolar concentrations to substrate (Richter 1934) was added.

(2) *Colorimetric Method.*—Developed in the course of some unpublished work by J. E. Humpoletz to determine tyrosinase concentration. The reaction mixture was 1.0 ml. of enzyme plus substrate and inhibitor and 0.001M phosphate buffer of pH 7.5 up to 10.0 ml. This was immediately examined in an E.E.L. photoelectric colorimeter using a blue filter (No. 303) and the optical density recorded every 15 sec. for 3-5 min. This was done at room temperature, about 22°C. When the optical density was plotted against time, a straight line was obtained, at least for the period ½-2½ min. The velocity of the reaction was recorded as the increase in optical density multiplied by 100 per min.

(3) *Chronometric Method*.—This was used by Miller *et al.* (1944). The reaction mixture was 2.0 ml. of enzyme plus substrate, inhibitor, and ascorbic acid, made up to 100 ml. with 0.001M phosphate buffer of pH 7.5. The action took place at room temperature, about 22°C. The velocity of the reaction was measured in ml. of ascorbic acid solution oxidized per sec.

III. RESULTS

(a) *Examination of Methods of Measuring Tyrosinase Activity*

As with previous workers (see Nelson and Dawson 1944, p. 117), difficulty experienced in maintaining a reasonably constant rate of oxygen uptake in the manometric method prevented accurate estimation of the initial reaction rate. Addition of gelatin or of *o*-phenylene diamine lessened but did not eliminate this difficulty; in the latter case some inhibition was also noted. Under no conditions could strictly linear relationship between enzyme concentration and initial velocity be obtained, even at extreme dilutions of enzyme and moderate substrate concentration (1mM catechol); this is undesirable for kinetic studies. Further, this technique measures the uptake of oxygen in at least two steps (Nelson and Dawson 1944), so that the rate measured is the combination of several rates, of which not all may be under enzymic control. Because of these difficulties, the technique was abandoned.

Preliminary experiments using the colorimetric technique and *p*-cresol, catechol, and pyrogallol as substrates at concentrations of 0.02-0.05mM and sodium *m*-hydroxybenzoate, phloroglucinol, and KCN as inhibitors and the potato enzyme gave apparently satisfactory figures in accordance with theory, showing sodium *m*-hydroxybenzoate and phloroglucinol as competitive inhibitors and KCN as a non-competitive inhibitor. Apparent Michaelis constants K_s^* for each substrate were of the order 0.1-0.5mM and inhibitor dissociation constants K_i for the competitive inhibitors were of the order 0.3-2.0mM.

However, when the substrate concentrations were increased serious anomalies and side effects were noted. Using catechol as substrate, the graph of velocity against substrate concentration did not become progressively flatter as the substrate concentration was raised above 1.0mM, as would be expected for a typical Michaelis curve, but it approximated to a straight line with a marked slope (Fig. 1). At the same time, some qualitative differences in the colour developed were noticed.

This colour is due to the combined effect of several compounds: the primary and, particularly, the secondary oxidation products of the substrate and their compounds with other substances in the medium, such as amino and imino acids (Jackson and Kendal 1949; Trautner and Roberts 1950). Differences in the reaction mixture, such as those between the potato enzyme with

* The following symbols are used throughout: E = enzyme concentration; S = substrate concentration; I = inhibitor concentration; v = velocity of the reaction; V = velocity of the reaction when the enzyme is saturated with substrate; K_s = Michaelis constant, the dissociation constant of the enzyme-substrate complex; K_i = dissociation constant of the enzyme-inhibitor complex.

its accompanying impurities and the relatively pure mushroom enzyme, might be expected to (and in fact were found to) lead to differences in the colour developed. But it was also noticed that, concurrent with the change from apparent conformity with Michaelis' theory to obvious non-conformity, increasing substrate concentration led to a qualitative change in colour. Between substrate concentrations of 1 and 5mM (with catechol) the colour changed progressively from red-brown to bright yellow; above 5mM, the colour remained qualitatively the same, though its rate of development increased approximately linearly. It should also be noted that, as with the manometric method, this technique measures components of several different reaction rates simultaneously. These considerations necessitated the abandonment of the method despite its very considerable saving of time compared with other methods of measuring tyrosinase activity.

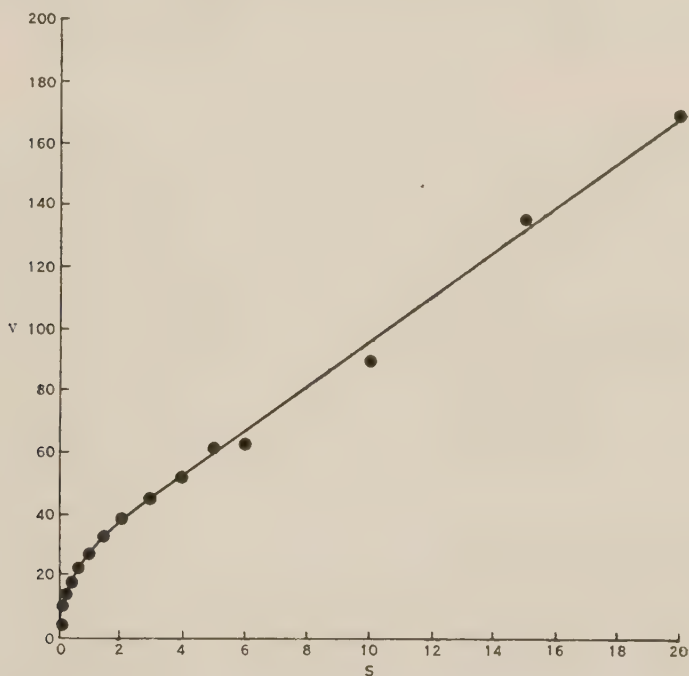


Fig. 1.—Relationship between enzyme action and substrate concentration for the potato preparation as measured by the colorimetric technique. Reaction velocity v measured in increase in optical density units per minute plotted against substrate concentrations expressed as millimolarity of catechol. The line was fitted by eye.

The chronometric technique has many advantages: it measures the rate of the primary oxidation of catechol to *o*-quinone, and gives a close approximation to the initial rate. However, it will measure only fairly rapid reaction rates within a limited range. The method would be more suitable to kinetic

studies if a more sensitive method for the detection of *o*-benzoquinone were available. It was, however, obviously the method of choice for the experiments that follow.

It is of interest to note that the colour developed in the reaction mixture after the oxidation of the ascorbic acid had taken place was qualitatively different from that developed in a similar mixture without the ascorbic acid.

(b) Kinetic Studies

Results for the potato enzyme are recorded graphically in Figure 2, as velocity against substrate concentration (Fig. 2A) and according to the first modification of Lineweaver and Burk (1934), as $1/v$ against $1/S$ (Fig. 2B). It proved difficult to get reproducible results using this enzyme source and only the most reliable results are plotted. More satisfactory results were obtained for the mushroom enzyme, and the data for a typical experiment are plotted in similar fashion in Figure 3.

IV. DISCUSSION

Theoretically, it is to be expected with competitive inhibition that increasing the concentration of substrate will decrease the proportionate inhibition by a given concentration of inhibitor, so that a concentration of substrate should be reached at which the velocity approximates to the maximum velocity obtained in the inhibitor's absence. Experimental limitations, as in the present work, often prevent the point being tested, but the treatment of Lineweaver and Burk (1934) can be readily applied. According to their first modification, when $1/v$ is plotted against $1/S$, the lines with and without inhibitor should have the same intercept, namely $1/V$, when the inhibition is competitive. Non-competitive inhibitors will give lines with intercepts and slopes increased in the same proportion, the intercept becoming $1/V (1 + I/K_i)$, and the slope having the same formula as with a competitive inhibitor, namely $K_s/V (1 + I/K_i)$, to be compared with the slope in the absence of inhibitor, K_s/V .

Working with resorcinol as inhibitor and an enzyme preparation from apples, the general trend of the figures obtained by Hackney (1948) for percentage inhibition on increasing substrate concentration would indicate some element of competition, but her Figure 10, plotted according to the treatment of Lineweaver and Burk, does not demonstrate simple competitive inhibition, in that the intercept does not remain unchanged when inhibitor is present. She calculates K_i as 0.000002 (presumably molar). It has been difficult to check this calculation as the different sets of data yield different values for the Michaelis constant (not noted by Hackney), and, as noted above, the theoretical maximum velocity is different in the presence and absence of inhibitor. However, using average values, the present author re-calculates K_i as of the order 0.1M, a value that invalidates Hackney's discussion of some observed anomalies in the competitive behaviour of resorcinol.

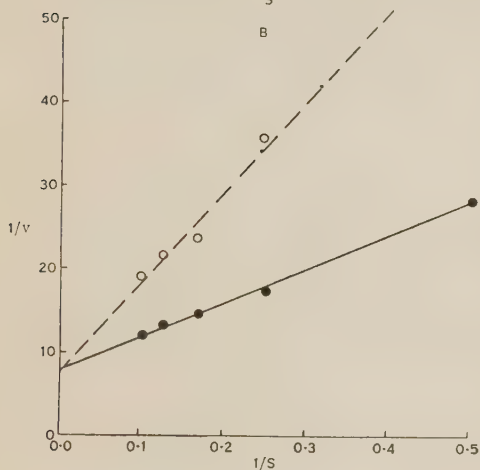
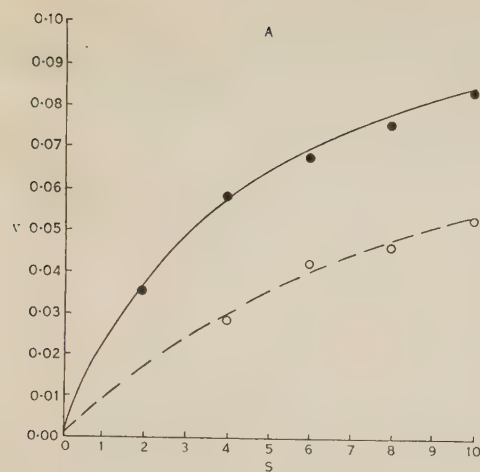


Fig. 2

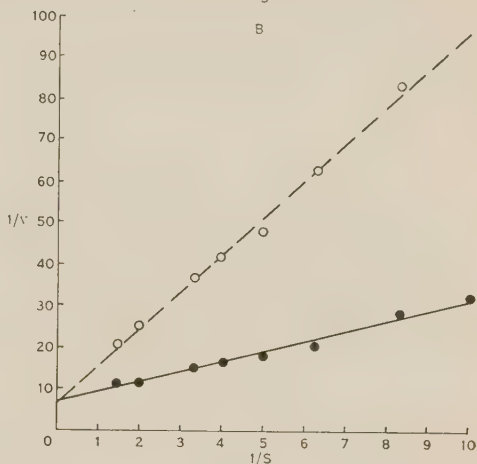
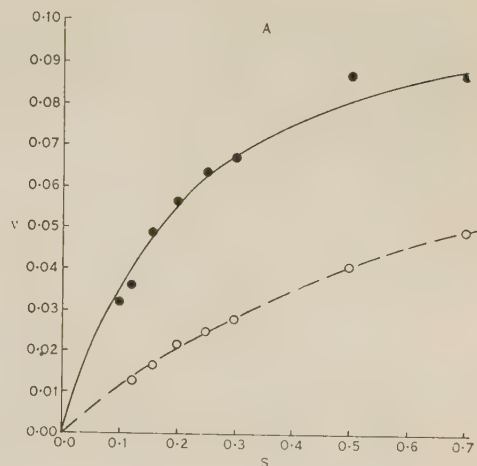


Fig. 3

Fig. 2.—Kinetics of the action of the potato preparation in the presence and absence of 4.0mM sodium *m*-hydroxybenzoate. The reaction velocities v were measured by the chronometric method and expressed in ml. of ascorbic acid solution oxidized per sec. The substrate was catechol and its concentration S expressed as millimolarity. Full lines, uninhibited enzyme; broken lines, inhibited enzyme.

A: Reaction velocity plotted against substrate concentration. Lines drawn through points calculated from Figure 2B.

B: Lineweaver and Burk graphs, $1/v$ plotted against $1/S$. Lines fitted by the least squares method.

Fig. 3.—Kinetics of the action of the mushroom preparation in the presence and absence of 2.0mM sodium *m*-hydroxybenzoate. The reaction velocities v were measured by the chronometric method and expressed in ml. of ascorbic acid solution oxidized per sec. The substrate was catechol and its concentration S expressed as millimolarity. Full lines, uninhibited enzyme; broken lines, inhibited enzyme.

A: Reaction velocity plotted against substrate concentration. Lines drawn through points calculated from Figure 3B.

B: Lineweaver and Burk graphs, $1/v$ plotted against $1/S$. Lines fitted by the least squares method.

It may be noted that Hackney's expectation that, with a non-competitive inhibitor, percentage inhibition would show a linear relationship with inhibitor concentration is in error. It can be readily shown that the curves for competitive and non-competitive inhibitors are both rectangular hyperbolae. Indeed, Hackney's own figures for a non-competitive inhibitor, KCN, as shown in her Table 4, give a curve and not a straight line if plotted in this fashion.

In the present work, it will be seen that sodium *m*-hydroxybenzoate meets the requirements of a competitive inhibitor of tyrosinase performing the activation of catechol, with the constants:

	Potato Enzyme	Mushroom Enzyme
Michaelis constant K_s	about 5mM	0.28mM
K_i	about 2.5mM	0.6mM

The different dissociation constants found for the enzyme prepared from potato and that from mushroom would indicate that either the enzymes had different properties or possibly the preparation from the potato contained a competitive inhibitor of tyrosinase that was lacking in the relatively pure mushroom preparation.

V. ACKNOWLEDGMENTS

Grateful acknowledgment is made to Mr. J. M. Vincent, Senior Lecturer in Agricultural Microbiology, University of Sydney, for his encouragement and advice during the course of this work, and for his assistance in preparing the manuscript. Acknowledgments are also due to Mr. D. A. Lewis for conducting the manometric tests and to Mr. J. F. Humpolett for advice concerning the colorimetric technique.

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VI. REFERENCES

- HACKNEY, F. M. W. (1948).—*Proc. Linn. Soc. N.S.W.* 73: 439.
 JACKSON, H., and KENDAL, L. P. (1949).—*Biochem. J.* 44: 477.
 LINEWEAVER, H., and BURK, D. (1934).—*J. Amer. Chem. Soc.* 56: 658.
 MICHAELIS, L., and MENTEN, M. L. (1913).—*Biochem. Z.* 49: 333.
 MILLER, W. H., MALLETT, M. F., ROTH, L. J., and DAWSON, C. R. (1944).—*J. Amer. Chem. Soc.* 66: 514.
 NELSON, J. M., and DAWSON, C. R. (1944).—*Adv. Enzymol.* 4: 100.
 ONSLOW, M. W. (1931).—"The Principles of Plant Biochemistry." (Cambridge University Press.)
 RICHTER, D. (1934).—*Biochem. J.* 28: 901.
 TRAUTNER, E. M., and ROBERTS, E. A. H. (1950).—*Aust. J. Sci. Res. B* 3: 356.

CONDITIONS AFFECTING THE ACTION OF FLUOROACETATE ON THE METABOLISM OF NEMATODE PARASITES AND VERTEBRATE ANIMALS

By V. MASSEY*† and W. P. ROGERS†

[Manuscript received April 20, 1951]

Summary

The respiration of brei or mince prepared from pigeon breast muscle containing 0.0003M methylene blue was very little affected by 0.01M fluoroacetate. Under similar conditions, the respiration of preparations from *Ascaridia galli* and *Nematodirus* spp. was inhibited 35-50 per cent. This inhibition was lessened by adding intermediates of the tricarboxylic acid cycle. Succinate was most effective in lifting the inhibition; oxaloacetate was least effective. The formation of citrate in brei of *Nematodirus*, containing methylene blue and added acetate and oxaloacetate, was increased more than 100 per cent. when fluoroacetate, 0.01M, was present. The utilization of citrate in mince prepared from *Nematodirus* containing 0.0003M methylene blue was strongly inhibited by 0.01M fluoroacetate when a gas phase of air was used; under anaerobic conditions no inhibition was found. Similar preparations from pigeon liver and pigeon breast muscle were inhibited only to a small degree, about 20 per cent., under aerobic conditions, and not at all under anaerobic conditions. When methylene blue was not added, the inhibition of citrate utilization in pigeon breast muscle preparations in air rose to about 50 per cent.; under oxygen the inhibition rose to 100 per cent. When cytochrome *c*, 5×10^{-6} M, was added, the inhibition was 100 per cent., even in a gas phase of air.

The inhibition of citrate utilization in mince prepared from pigeon breast muscle was examined at different oxidation-reduction potentials maintained by electrolysis. At Eh levels below +50 mV., 0.01M fluoroacetate caused inhibitions of 0-25 per cent.; at levels above +120 mV. the inhibition ranged from 65 to 100 per cent.

The possibility that it was necessary for fluoroacetate to be metabolized for a period before inhibition occurred was also examined. Mince prepared from pigeon breast muscle, to which was added 0.025M oxaloacetate and fluoroacetate, was pre-incubated for 15 min. under oxygen, or under oxygen-free nitrogen. Thereafter, the inhibition of citrate utilization occurring under air, oxygen, or nitrogen during a period of 30 min. was determined. Inhibition in air after pre-incubation in nitrogen was 20-30 per cent.; after pre-incubation in oxygen, 80-90 per cent. After pre-incubation in oxygen, inhibition in nitrogen was nil, and in oxygen, 100 per cent.

It is suggested that these results support the hypothesis that fluoroacetate condenses with oxaloacetate to form the actual inhibitor. Both the formation of the inhibitor and the inhibiting reaction were increased in the presence of oxygen.

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I. INTRODUCTION

It would appear that small species of nematode parasites inhabiting the small intestine of host animals are at least partially aerobic *in vivo* (Rogers 1949). In these organisms aerobic energy is probably obtained from the reactions of the tricarboxylic acid cycle (Massey and Rogers 1950*a*). Acetate is rapidly metabolized by the parasites, condensing, either as acetate or a closely related compound, with oxaloacetate to form citrate (Massey and Rogers, unpublished data). The early report that fluoroacetate could be regarded as a competitive inhibitor of biochemical reactions involving acetate (Bartlett and Barron 1947) led us to examine and compare its action on the metabolism of nematode parasites and host animals. The results of these experiments showed that it would be necessary to examine the effect of changes in oxidation-reduction potentials on the efficiency of fluoroacetate as an inhibitor and to examine the hypothesis advanced by Liebecq and Peters (1949) on the mode of action of the poison.

According to these authors, fluoroacetate itself is not an inhibitor, but it is activated and brought into the tricarboxylic acid cycle with the formation of fluoro derivatives of one or more of the normal components of the cycle. The fluoro compounds, it was suggested, cause a "jamming" of the cycle that leads to the accumulation of citrate. The results of an examination of some aspects of this hypothesis, together with a study of conditions affecting the efficiency of fluoroacetate, are reported in this paper.

The nematodes examined were *Nematodirus filicollis*, *N. spathiger*, and *Ascaridia galli*. For comparing vertebrate tissues with those of nematodes, pigeon breast muscle and pigeon liver were selected. These cannot be regarded as being truly representative of "host" tissues; they were selected as much-studied materials of which something of the metabolism is known.

II. MATERIALS AND METHODS

Nematodirus filicollis and *N. spathiger*, which were not separated for use, were obtained from naturally infested sheep; *Ascaridia galli* was obtained from experimentally infected chickens. The parasites were separated from the hosts' intestinal contents and washed with saline, and then ground with quartz or minced in the mincer of the type described by Seevers and Shideman (1941). Pigeon breast muscle and pigeon liver were similarly treated. Unless stated otherwise, the tissue preparations were diluted with Krebs-Ringer phosphate buffer at pH 7.3 without calcium chloride. All materials added during experiments were brought to the appropriate pH.

Some of the fluoroacetic acid used in these experiments was obtained from Dr. B. C. Saunders, of the University of Cambridge; the remainder was prepared from commercial sodium fluoroacetate by acidifying and distilling at reduced pressure. Both fluoroacetate preparations had similar effects on biological systems.

Oxygen consumption was measured by the "direct" method of Warburg (1926). Citrate was determined by the Krebs and Eggleston (1944) modifica-

tion of the method of Pucher, Sherman, and Vickery (1936). In initial experiments the colorimetric method was used; later determinations were made by titration, which, though more time-consuming, was more accurate and consistent. Cytochrome *c* was prepared by the method of Keilin and Hartree (1937).

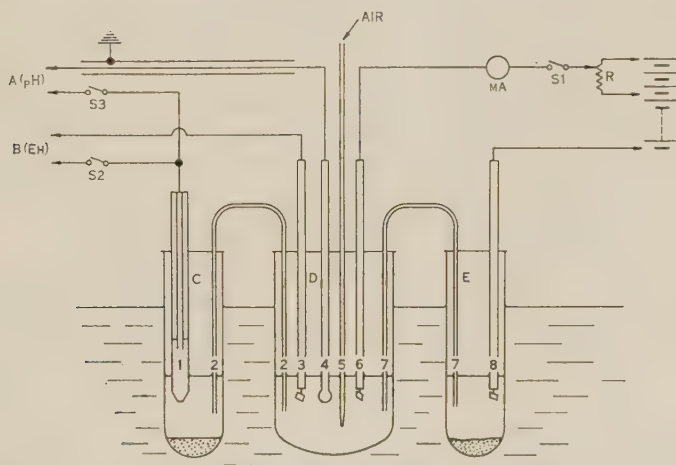


Fig. 1.—Apparatus used for controlling oxidation-reduction potentials by electrolysis. For explanation, see text.

Experiments involving the control of oxidation-reduction potentials were carried out in vessels shown in Figure 1. The potential was controlled by electrolysis as described by Hanke and Katz (1943). Vessel C contained saturated potassium chloride into which dipped the saturated calomel electrode, 1, and the potassium chloride-agar bridge, 2. The cell, D, was the reaction vessel in which the brei was incubated. It contained an arm of the bridge, 2; a bright platinum electrode, 3, for *Eh* determinations; a glass electrode, 4, for pH determinations; a glass tube, 5, from which air was bubbled through the brei; a platinum electrode, 6, and an arm of the sodium chloride- or sodium ammonium hydrogen phosphate-agar bridge to conduct the electrolysis current. Vessel E took the other arm of the bridge, 7, and a platinum electrode, 8, in contact with saturated sodium chloride, to complete the electrolysis current. Oxidation-reduction potentials and pH were read on separate potentiometers. When not in use, the glass electrode was removed from contact with the brei in cell D. The electrolysis current was provided by accumulators through the variable resistance, *R*, and the switch, *S*₁. The system of vessels and electrodes was duplicated and supported in a transformer oil-bath at 38°C.

The action of electrolysis in controlling the oxidation-reduction potential was tested by alternately oxidizing and reducing suitable indicators and observing the relation between colour changes and potential. Methylene blue (*E*₀ at pH 7.0, + 11 mV.), thionine (+ 63 mV.), toluyene blue (+ 115 mV.), and 2:6-dichlorophenolindophenol (+ 217 mV.) were examined in this manner. It was found that, starting with the dyes reduced with a little sodium

dithionite, oxidation and reduction could be controlled by electrolysis and the colours at different oxidation-reduction potentials agreed generally with those given by Hewitt (1950).

Before commencing an experiment, the electrodes for measuring potentials were tested by using quinhydrone in phthalate buffer and in 0.1N HCl. The error for the electrodes in both cells was never greater than 5 per cent. and was usually less than 2 per cent.

It should be noted that the potentials recorded on the platinum electrodes during experiments with biological preparations were not necessarily those of the "partial oxidation-reduction potentials" of the enzyme systems under examination. This error would not seriously affect the conclusions drawn from the results of the experiments.

The control of the oxidation-reduction potential of brei by electrolysis was found to be difficult. Only after a number of trials was it found possible to keep the Eh at within ± 10 per cent. of a required value. At times, though usually for periods of less than a minute, the potential of one or other of the two cells varied by as much as ± 20 per cent. from the required value. In the experiments described later in this paper the Eh was maintained at certain levels largely within ± 10 per cent. for periods of 30 min. by noting the E_1 every 3 min. and modifying the electrolysis current accordingly. Variations of more than ± 10 per cent. occurred only for very short periods.

The utilization of citrate in the biological preparations varied in different experiments, even when the Eh was kept constant. However, when aliquots from the one lot of mince were used and the Eh was kept at the same value in the two cells, the variation in the utilization of citrate was less than 8 per cent. for the three experiments carried out.

During the final experiments the mince in the two reaction cells was kept as near as possible to the selected Eh; one cell was used for fluoroacetate-poisoned tissue, and the other for the control. The mince in the cells was stirred by streams of air bubbles; the air for the fluoroacetate-poisoned preparation was passed through 0.1M phosphate buffer at pH 7.3 at 38°C. to which fluoroacetate was added to give the same concentration as that in the mince. Air from the control cell was passed through phosphate buffer only.

The separation of the components of the tricarboxylic acid cycle was accomplished by the paper partition chromatographic method of Lugg and Overell (1948). The phase mixtures found most suitable for separating the acids were amyl alcohol-water, with formic acid to a concentration of 5N in the water, and mesityl oxide-water, again with 5N formic acid.

The biological material was prepared for chromatography by precipitating the proteins with sodium tungstate and sulphuric acid, followed by continuous extraction of the protein-free filtrate with ether for 48 hr. The ether was then evaporated off and the residue dissolved in water. After adjusting the pH to 2.2 this solution was then applied to the filter paper for chromatographic separation.

Fluorine in the compounds which had been separated by paper chromatography was determined colorimetrically. Appropriate pieces were cut from the chromatogram and any organic fluoride present was converted to the inorganic form by fusing with metallic sodium at 400°C. *in vacuo* for one hour. Fluorine was freed from the digest by steam distillation after the addition of perchloric acid. The fluoride in the distillate was estimated by the zirconium-alizarin lake method of Scott (1941). Areas from the chromatogram that had been traversed by the developing fluid were used for blank determinations. By this method the fluorine in 20 μ g. fluoroacetic acid placed on filter paper could be detected.

TABLE I
EFFECT OF FLUOROACETATE ON OXYGEN UPTAKE

Biological Material	Inhibition Produced by Fluoroacetate	
	0.01M	0.02M
<i>Nematodirus</i> spp.	30-65%	
Mean	55% (10)	—
<i>Ascaridia galli</i>	20-45%	
Mean	35% (8)	—
Pigeon breast muscle	0%	0-10%
Mean	0% (8)	5% (6)

The figures within brackets refer to the number of experiments from which the mean results were calculated. The tissue was used as a brei suspended in Krebs-Ringer phosphate solution at pH 7.3, and contained methylene blue at a final concentration of 0.0003M.

III. PROCEDURE AND RESULTS

(a) Effect of Fluoroacetate on Oxygen Consumption

The action of fluoroacetate on the oxygen uptake of brei prepared from *Nematodirus* spp., *Ascaridia galli*, and pigeon breast muscle was examined at concentrations of 0.01M and 0.02M in Warburg manometers. All preparations contained methylene blue at a concentration of 0.0003M. The brei was in contact with the inhibitor during the preliminary 10 min. equilibration period before the taps were closed. The results given in Table 1 were calculated from the figures obtained during the first 15 min. of the experiment. Inhibition in pigeon breast muscle was small or absent even at the higher fluoroacetate concentration, whereas in the brei prepared from the parasites it was usually in the region of 34-45 per cent. at low fluoroacetate concentrations.

(b) Effect of Intermediates of the Tricarboxylic Acid Cycle on Fluoroacetate Inhibition

The effect of α -ketoglutarate, succinate, fumarate, malate, and oxaloacetate (0.01M) on the oxygen uptake of *Nematodirus* spp. and of *A. galli* brei in the absence of, and in the presence of, 0.01M fluoroacetate was determined in Warburg manometers. As before, the inhibitor, together with the substrate, was

present with the brei during the equilibration period, and the results (Table 2) were obtained from the first 15 min. of the experiments. Similar results were obtained when the experiment was repeated several times. All the substrates lessened the fluoroacetate inhibition; succinate was most effective and oxaloacetate the least effective in this regard.

TABLE 2
EFFECT OF SUBSTRATES ON FLUOROACETATE INHIBITION

	<i>Nematodirus</i> spp.		<i>Ascaridia galli</i>	
	Oxygen Uptake (μ l.)	Inhibition (%)	Oxygen Uptake (μ l.)	Inhibition (%)
No additions	— 160		— 115	
+ Fluoroacetate	— 70	56	— 70	39
+ α -Ketoglutarate	— 205		— 150	
+ α -Ketoglutarate + fluoroacetate	} — 140	32	— 150	0
+ Succinate	— 290		— 205	
+ Succinate + fluoroacetate	} — 280	3	— 200	2
+ Fumarate	— 220		— 165	
+ Fumarate + fluoroacetate	} — 170	23	— 130	21
+ Malate	— 215		— 155	
+ Malate + fluoroacetate	} — 180	16	— 130	16
+ Oxaloacetate	— 180		— 170	
+ Oxaloacetate + fluoroacetate	} — 105	40	— 135	26

Each flask contained brei in Krebs-Ringer phosphate solution at pH 7.3 without calcium chloride and containing 0.0003M methylene blue. The substrates and the inhibitor were used at a final concentration of 0.01M. The results were obtained during the first 15 min. of the experiment.

(c) Effect of Fluoroacetate on Citrate Formation

Brei, prepared from *Nematodirus* spp. containing 0.0005M methylene blue, was shaken in Erlenmeyer flasks at 38°C. in air. Oxaloacetate, acetate, and fluoroacetate were added in different combinations to different flasks to give a concentration of 0.01M for each component. Samples for citrate determinations were taken after the flask contents had been shaken for a few minutes. One hour later samples were again taken for citrate determination. Typical results are shown in Table 3.

Though acetate and oxaloacetate did not stimulate citrate formation very much in the absence of fluoroacetate, the increase was very clear when the inhibitor was present. These experiments may be taken as showing that citrate formation in nematode parasites probably involves the condensation of oxaloacetate and acetate or a compound derived from acetate, and that fluoroacetate

(d) Effect of Fluoroacetate on Citrate Utilization

TABLE 3

System	Citrate Formation in 1 Hr. (mg.)
No addition	0.05
+ Oxaloacetate	0.05
+ Acetate	0.08
+ Oxaloacetate, acetate	0.50
+ Fluoroacetate	0.15
+ Oxaloacetate, acetate, fluoroacetate	1.12

The results of these experiments suggested that the efficiency of fluoroacetate inhibition might, in some way, be associated with the oxygen tension at which the experiment was conducted. This hypothesis was therefore examined.

(e) Effects of Oxygen Tension and Oxygen Carriers on Fluoroacetate Inhibition

Citrate was added to mince prepared from pigeon breast muscle in Krebs-Ringer phosphate without calcium chloride. Methylene blue, cytochrome *c*, and fluoroacetate were added to aliquots of the mince as shown in Table 5. Samples for citrate estimation were taken at the beginning of the experiment and after shaking in air for 2 hr. at 38°C. The results (Table 5) indicate that fluoroacetate was a much more effective inhibitor in the absence of methylene blue, and when cytochrome *c* was added. Similar results were obtained when these experiments were repeated, though in some cases the addition of cytochrome *c* caused an accumulation of citrate even in the absence of fluoroacetate. The accumulation was always increased in the presence of fluoroacetate, however.

TABLE 4
EFFECT OF FLUOROACETATE ON THE UTILIZATION OF CITRATE

System	Amount of Citrate Utilized (mg.)		
	<i>Nematodirus</i> spp.	Pigeon Breast Muscle	Pigeon Liver
No additions, aerobic	1.80	1.65	1.20
+ Fluoroacetate, 0.01M, aerobic	Nil	1.40	1.14
No additions, anaerobic	1.55	1.50	1.10
+ Fluoroacetate, 0.01M, anaerobic	1.46	1.47	1.10

Each flask contained 2 mg. citrate in 5 ml. of mince suspended in Krebs-Ringer phosphate solution at pH 7.3 without calcium chloride. Methylene blue (final conc. 0.0005M) was present. The figures show the utilization of citrate during shaking at 38°C. for 2 hr. Anaerobic conditions were established by gassing with oxygen-free nitrogen and absorbing any residual oxygen in the flasks with yellow phosphorus.

The fluoroacetate inhibition of citrate utilization in the absence of methylene blue was then examined at different oxygen tensions. After taking samples for the determination of the initial citrate concentrations, anaerobic conditions were established in some flasks, others were left open to the air, and others were gassed with oxygen for 5 min. before closing the taps. The flasks were shaken in a water bath for 40 or 60 min. at 35°C. after which samples were taken for the determination of the final citrate concentrations. The results of one set of experiments are shown in Table 6. It should be noted that the fluoroacetate concentration in the last group of experiments was 0.005M. The lower concentration was used because citrate was often found to accumulate when oxygen was used as the gas phase, even when fluoroacetate was not present. The inhibitor always increased the accumulation of citrate, however.

The results shown in Table 6 support the suggestion that the efficiency of fluoroacetate is associated with the oxygen tension at which the experiment is carried out. This hypothesis was then taken further by examining fluoroacetate inhibition of citrate utilization at different oxidation-reduction potentials.

TABLE 5
EFFECT OF METHYLENE BLUE AND CYTOCHROME *c* ON FLUOROACETATE INHIBITION
OF THE UTILIZATION OF CITRATE IN PIGEON BREAST MUSCLE

System	Initial Citrate (mg.)	Final Citrate (mg.)	Inhibition (%)
No additions	1.20	0.65	
+ Fluoroacetate, 0.01M	1.20	0.95	55
+ Methylene blue	1.20	0.65	
+ Fluoroacetate, 0.01M, methylene blue	1.20	0.80	27
+ Cytochrome <i>c</i>	1.20	0.95	
+ Cytochrome <i>c</i> , fluoroacetate, 0.01M	1.20	1.20	100

Each flask contained 5 ml. of mince suspended in Krebs-Ringer phosphate solution without calcium chloride at pH 7.3. When methylene blue was added, the final concentration was 0.001M; the added cytochrome *c* gave a concentration of 5×10^{-6} M. The figures show the amounts of citrate present at the beginning of the experiment and after shaking the flasks at 38°C. for 2 hr.

(f) *Fluoroacetate Activity at Different Oxidation-Reduction Potentials*

Experiments were carried out with mince prepared from pigeon breast muscle in Krebs-Ringer phosphate solution at pH 7.3 without calcium chloride. The phosphate-buffer concentration was increased to give a final concentration of 0.05M. Methylene blue, 0.0004M, and citrate, 1 mg./ml., were also present. Fluoroacetate was used at a final concentration of 0.01M. A small amount of octyl alcohol was added to the mince from time to time to prevent frothing caused by the aeration. The utilization of citrate was not affected by small amounts of octyl alcohol.

In preliminary experiments the pH of the mince was adjusted to 7.6 when positive electrolysis was to be used, and to pH 7.0 for negative electrolysis. No great care was taken to keep the equilibration period constant or to prevent the loss of fluoroacetate from the mince caused by aeration. The experiments were carried out for an hour after the desired Eh had been obtained. During the experiment pH changes of the order of 0.6 took place when extreme oxidation-reduction potentials were used. Five experiments were carried out to determine the effect of fluoroacetate at Eh's below + 50 mV. Inhibition varied from nil to 25 per cent. In 12 experiments carried out at Eh's above + 150 mV. the inhibition varied between 65 and 100 per cent. One discordant result was obtained when no inhibition was found at + 250 mV.

In the final experiments, the technique was modified to maintain the equilibration period during which the required oxidation-reduction potential was attained, at 30 ± 5 min. At the end of the equilibration period fluoroacetate in buffer was added to the mince in one of the cells and buffer only to the other. Fluoroacetate was also added to the dilute buffer at 38°C . through which the air was passed before being bubbled through the mince. The experimental period was reduced to 30 min. Under these circumstances the removal of fluoroacetic acid from the poisoned mince by aeration was probably very slow, and the changes in pH caused by the electrolysis were much smaller, so that the divergence from pH 7.0, to which the mince was adjusted at the beginning of the equilibration period, was small. The pH was determined at the beginning and end of the experiment, the Eh was read at intervals of 3 min. and the electrolysis current modified accordingly. The results of these experiments are shown in Table 7.

TABLE 6
EFFECT OF OXYGEN TENSION ON FLUOROACETATE INHIBITION OF THE UTILIZATION OF CITRATE IN PIGEON BREAST MUSCLE

		Duration of the Experiment (min.)	Initial Citrate (mg.)	Final Citrate (mg.)	Inhibition (%)
Gas Phase					
No additions	}	Nitrogen	60	1.30	Nil
+ Fluoroacetate, 0.01M				0.66	
No additions	}	Air	60	1.30	47
+ Fluoroacetate, 0.01M				0.65	
No additions	}	Oxygen	40	1.30	70
+ Fluoroacetate, 0.005M				0.55	
No additions	}	Air	40	1.16	32
+ Fluoroacetate, 0.005M				0.59	
No additions	}	Air	40	1.16	32
+ Fluoroacetate, 0.005M				1.00	
No additions	}	Air	40	1.32	32
+ Fluoroacetate, 0.005M				0.57	
No additions	}	Air	40	1.32	32
+ Fluoroacetate, 0.005M				0.80	

Each flask contained 5 ml. of mince suspended in Krebs-Ringer phosphate solution at pH 7.3 without calcium chloride.

In a proportion of the experiments the utilization of citrate was small. The results of these experiments were discarded because inhibitions calculated from such results might have been inaccurate. In general, it was found that the utilization of citrate at high oxidation-reduction potentials was smaller than at low Eh's. At very high potentials fluoroacetate caused an accumulation of citrate.

(g) *Nature of Fluoroacetate Inhibition*

According to the hypothesis advanced by Liebecq and Peters (1949), there would be two phases in the action of fluoroacetate. First would come the formation of a fluoro compound, possibly fluorocitrate, followed by the inhibition arising from the action of this compound. Experiments were carried out to see if one or other of these phases was particularly associated with Eh conditions. Accordingly, attempts were made to determine whether the "fluorocitrate" was formed and to see if its formation was affected by the oxidation-reduction potential. The first part of the experiment was carried out using paper chromatography as a means of separating the "fluorocitrate." In the second part, the effect of the oxygen tension during a pre-incubation period on the subsequent inhibition of citrate utilization was examined.

TABLE 7
EFFECT OF OXIDATION-REDUCTION POTENTIAL ON FLUOROACETATE INHIBITION ON THE UTILIZATION OF CITRATE IN PIGEON BREAST MUSCLE

Eh (mV. at pH 7.2, 38°C.)		Inhibition (%)	Change in pH
Control	Fluoroacetate		
44	49	4	7.0 — 7.3
82	73	Nil	7.0 — 7.2
89	90	Nil	7.0 — 7.2
128	132	100	7.0 — 7.1
170	158	70	7.0 — 7.1
170	178	100	7.0 — 7.1

The Eh figures give the average potential maintained over 30 min. during which the utilization of citrate was measured. The changes in pH occurring during the experiments are also shown.

Mince prepared from *Nematodirus* spp. and pigeon breast muscle to which oxaloacetate had been added was incubated, with and without fluoroacetate. By means of the paper chromatographic method of Lugg and Overell (1948), the tricarboxylic acid cycle components were separated. Citric, α -ketoglutaric, succinic, malic, fumaric, and oxaloacetic acids could be identified. Fluorine in the "citrate" spots obtained from mince which had been incubated with and without fluoroacetate was then determined. At times fluorine in the "citrate" spots from fluoroacetate-treated material was up to 5 μ g. more than in the spot from the untreated mince. These results were not consistent, however, and no definite conclusions could be drawn from the experiments.

Since this work was carried out, Buffa *et al.* (1950) have reported that they have isolated, by chromatography from guinea pig kidney homogenates treated with fluoroacetate, a tricarboxylic fraction containing a small amount of fluorine that is active in preventing the utilization of added citrate. The failure to obtain a consistent separation of the fluoro compound in the present investigation was most probably due to the insensitivity of the methods used.

In the second part of the experiments on the nature of fluoroacetate inhibition, mince was prepared from pigeon breast muscle and citrate was added to a concentration of about 1 mg./ml. Oxaloacetate, 0.0025M, with and with-

out fluoroacetate, 0.0025M, was added and the mixture was incubated for 15 min. under oxygen or oxygen-free nitrogen. Thereafter, the inhibition of citrate utilization was determined over a period of 30 min., the gas phase being oxygen-free nitrogen, air, or oxygen. The results are shown in Table 8. This experiment was repeated several times and consistently showed that inhibition was much greater when oxygen was present, both during the pre-incubation and the experimental periods. The finding that the pre-treatment of the mince influenced the amount of inhibitor present supports the suggestion that fluoroacetate was changed in some way before the inhibition took place.

TABLE 8
EFFECT OF OXYGEN TENSION ON FLUOROACETATE INHIBITION OF THE UTILIZATION OF CITRATE IN PIGEON BREAST MUSCLE

Gas phase, pre-incubation period	N ₂	O ₂	O ₂	O ₂
Gas phase, experimental period	Air	Air	O ₂	N ₂
Inhibition of citrate utilization (%)	26	86	100	Nil

The mince in Krebs-Ringer phosphate without calcium chloride contained 0.0025M oxaloacetate, with and without 0.0025M fluoroacetate, and was incubated for 15 min. under the different gases before the experiment commenced. The inhibition of the utilization of citrate occurring under the different gases was determined over 30 min.

IV. DISCUSSION

One of the most characteristic actions of fluoroacetate is its inhibition of the utilization of citrate both *in vitro* (Kalnitsky 1948; Liebecq and Peters 1949; Massey and Rogers 1950*b*) and *in vivo* (Buffa and Peters 1949). The increased yields of citrate obtained from the condensation of acetate and oxaloacetate in biological systems poisoned with fluoroacetate (Kalnitsky 1949; Massey and Rogers 1950*b*) is an outcome of this action. Recent work (Buffa *et al.* 1950; Elliott and Kalnitsky 1950) has indicated that fluoroacetate, when incubated with the appropriate biological preparation, undergoes condensation with oxaloacetate to form a fluoro compound, probably fluorocitrate, which may be separated by chromatography or by precipitation with calcium. The inhibition leading to the accumulation of citrate may be attributed to this compound but it has no effect on aconitase, isocitric dehydrogenase, or oxalosuccinic decarboxylase in isolated systems (Buffa *et al.* 1950). The results of the present investigation, which supports and enlarges these findings, are discussed below.

(1) The action of fluoroacetate on the metabolism of preparations from certain nematode parasites was characterized by the inhibition of respiration and of citrate utilization, and by the increased accumulation of citrate formed by the condensation of acetate and oxaloacetate. The nematode parasites concerned are able to oxidize pyruvate via the tricarboxylic acid cycle (Massey and Rogers 1950*a*). It may be said, then, that fluoroacetate has a similar action on the microorganisms, the vertebrate tissues, and the nematode parasites that it poisons.

(2) The present investigation has shown that the efficiency of fluoroacetate as an inhibitor of the utilization of citrate is affected by the nature of the

oxygen carrier present, the oxygen tension, and the oxidation-reduction potential maintained by electrolysis. The action of the carriers, methylene blue (E_0 , + 11 mV. at pH 7.0), which decreased the inhibition, and cytochrome *c* (E_0 , + 270 mV. at pH 7.4) (Ball 1938), which increased inhibition, was paralleled by that of the oxidation-reduction potential controlled by electrolysis or by varying the gas phase, and it is probable that the potential changes were the fundamental factors affecting the activity of the drug. It seems unlikely that the results were obtained by direct toxic action of the agents used to influence the oxidation-reduction potential. Nor would it appear that enzyme denaturation, which might have occurred at high potentials, would have appreciably affected the results.

The action of the oxidation-reduction potential in controlling enzyme activity is well established. Thus the activity of urease (Sizer and Tytell 1941) and yeast invertase (Sizer 1941) has been shown to be a function of the oxidation-reduction potential; the relative hydrolytic and synthetic activities of α - and β -amylase are affected by the potential (Ito 1939); and the actions of autolytic proteases are strongly affected by the oxidation-reduction potential (Reiss 1938). It appears, then, that one explanation of the action of the oxidation-reduction potential on fluoroacetate inhibition might be that different enzyme systems are concerned with the metabolism of citrate at high and low potentials. The system functioning at low potentials would be the one unaffected by the poison.

It is notable that active utilization of citrate in mince prepared from pigeon breast muscle was obtained under anaerobic conditions. This might have occurred partly by way of the tricarboxylic acid cycle, with coenzyme-linked reactions leading to electron and proton transfer to substrates other than oxygen. In the formation of citrate under anaerobic conditions from oxaloacetate and acetoacetate, Hunter and Leloir (1945) found that oxaloacetate acted as an oxidant and that α -ketoglutarate was oxidized. However, if a similar type of reaction was concerned in the utilization of citrate under anaerobic conditions, fluoroacetate might be expected to retain its inhibiting action if the actual inhibitor acted in competition with the normal substrate, citrate, or some closely allied compound.

(3) The two reactions that are probably concerned in fluoroacetate inhibition, the formation of the inhibitory compound, and the actual inhibiting reaction, may be further defined by the effect of the oxygen tension upon the two reactions. The results in Table 8 show that the degree of inhibition obtained was not immediately dependent on the amount of fluoroacetate present and that the potential inhibiting activity increased when the preparations were incubated aerobically. One explanation of these results would be that fluoroacetate condensed with oxaloacetate during the pre-incubation period, thus forming the inhibitor proper. It is uncertain whether this reaction would be expected to proceed in the absence of oxygen. Under certain circumstances the formation of citrate from acetoacetate and oxaloacetate can take place anaerobically (Breusch and Keskin 1944; Hunter and Leloir 1945). Kalnitsky

(1949) found that citrate formation from oxaloacetate in rabbit-kidney homogenates was decreased by 52-55 per cent. when incubated in nitrogen instead of air. However, fluoroacetate was present in the reaction mixture and the results obtained must have been influenced by the action of the oxidation-reduction potential on the activity of the inhibitor.

The second phase of the action of fluoroacetate on the utilization of citrate, the inhibition proper, was also strongest at high oxygen tensions (see Table 8). Buffa *et al.* (1950) failed to obtain inhibition with the "active" fluoro compound obtained from fluoroacetate in isolated systems of aconitase, *isocitric* dehydrogenase, and oxalosuccinic decarboxylase. It seems that the study of isolated systems should receive further consideration in relation to oxidation-reduction potentials favourable for the action of the inhibitor.

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VI. REFERENCES

- BALL, E. G. (1938).—*Biochem. Z.* **295**: 262.
 BARTLETT, G. R., and BARRON, E. S. G. (1947).—*J. Biol. Chem.* **170**: 67.
 BREUSCH, F. L., and KESKIN, H. (1944).—*Enzymologia* **11**: 243.
 BUFFA, P., LOTSPEICH, W. D., PETERS, R. A., and WAKELIN, P. W. (1950).—*Biochem. J.* **47**: xvii.
 BUFFA, P., and PETERS, R. A. (1949).—*Nature* **163**: 914.
 ELLIOTT, W. B., and KALNITSKY, G. (1950).—*Fed. Proc.* **9**: 168.
 HANKE, M. E., and KATZ, V. J. (1943).—*Arch. Biochem.* **2**: 183.
 HEWITT, L. F. (1950).—"Oxidation-Reduction Potentials in Bacteriology and Biochemistry." (F. and S. Livingstone Ltd.: Edinburgh.)
 HUNTER, F. E., and LELOIR, L. F. (1945).—*J. Biol. Chem.* **159**: 295.
 ITO, R. (1939).—*Fukuoka Acta Med.* **22**: 88.
 KALNITSKY, G. (1948).—*Arch. Biochem.* **17**: 403.
 KALNITSKY, G. (1949).—*J. Biol. Chem.* **179**: 1015.
 KEILIN, D., and HARTREE, E. F. (1937).—*Proc. Roy. Soc. B* **122**: 298.
 KREBS, H. A., and EGGLESTON, L. V. (1944).—*Biochem. J.* **38**: 426.
 LIEBECQ, C., and PETERS, R. A. (1949).—*Biochim. Biophys. Acta* **3**: 215.
 LUGG, J. W. H., and OVERELL, B. T. (1948).—*Aust. J. Sci. Res. A* **1**: 98.
 MASSEY, V., and ROGERS, W. P. (1950a).—*Aust. J. Sci. Res. B* **3**: 251.
 MASSEY, V., and ROGERS, W. P. (1950b).—*Nature* **165**: 681.
 PUCHER, G. W., SHERMAN, C. C., and VICKERY, H. B. (1936).—*J. Biol. Chem.* **113**: 235.
 REISS, P. (1938).—*C.R. Soc. Biol. Paris* **128**: 1179.
 ROGERS, W. P. (1949).—*Aust. J. Sci. Res. B* **2**: 166.
 SCOTT, R. D. (1941).—*J. Amer. Wat. Wks. Ass.* **33**: 2018.
 SEEVERS, M. H., and SHIDEMAN, F. E. (1941).—*Science* **94**: 351.
 SIZER, I. W. (1941).—*J. Gen. Physiol.* **25**: 399.
 SIZER, I. W., and TYTELL, A. A. (1941).—*J. Biol. Chem.* **138**: 631.
 WARBURG, O. (1926).—*Biochem. Z.* **164**: 481.

THE UPTAKE OF RADIO-ACTIVE PHOSPHATE BY NEMATODE PARASITES AND BY TISSUES OF THE SHEEP

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Summary

The relative uptake of radio-active phosphate by the tissues of the sheep and by the parasites *Trichostrongylus* spp., *Haemonchus contortus*, and *Oesophagostomum columbianum* has been investigated.

After injections into the abomasum of the host, *Trichostrongylus* accumulated phosphate very rapidly and in excess of the absorption by the small intestine of the host, but after intravenous injections the uptake by the parasites was similar to that of the small intestine.

The uptake of labelled phosphate by *H. contortus* showed an appreciable rise 8 hr. after both intra-abomasal and intravenous injections, whereas by this time the phosphate content of the host's abomasal tissues was decreasing.

O. columbianum absorbed less phosphate than the tissues of the host's rectum up to 4 hr. after both methods of dosing, but from that period showed a rise that was somewhat variable.

The significance of these results is discussed. It is suggested that the parasites feed on the tissues of the host and not on the contents of the alimentary tract.

I. INTRODUCTION

Wright (1950) has emphasized the necessity for further knowledge of the physiology of parasites as a basis for the development of more efficient anthelmintics. An understanding of the feeding habits of nematode parasites of the alimentary tract is of some importance in determining the mode of entry of anthelmintics into these parasites. It is of particular importance in assessing the mode of uptake of phenothiazine by parasites of the alimentary canal of sheep, and the present work has been undertaken partly in this connection.

Isotopic tracers have been used to follow the rates of absorption of substances by parasites attached to the mucous membrane of the host and by those free in the alimentary tract. Rogers and Lazarus (1949) have used labelled phosphate to show that *Ascaridia galli* feeds on the gut contents of the chicken, whereas *Nippostrongylus muris*, a parasite of the small intestine of the rat, is a tissue feeder. Read (1950) has used radio-active phosphorus in studying the uptake of phosphate and the carbohydrate metabolism of the cestode *Hymenolepis diminuta*.

Radio-active phosphorus has therefore been selected as a tracer for following the uptake of labelled phosphate by nematode parasites of the sheep and as a means of determining the nature of their feeding habits.

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II. MATERIALS AND METHODS

The nematodes examined in this investigation, *Haemonchus contortus*, *Trichostrongylus* spp. (very largely *T. colubriformis*), and *Oesophagostomum columbianum*, were obtained from naturally infested sheep.

Radio-active phosphorus was used as sodium dihydrogen phosphate in phosphate buffer at pH 7.3. The material was prepared for estimation by ashing with perchloric and nitric acids and precipitating the phosphate as magnesium ammonium phosphate with a magnesium citrate reagent (Mathison 1909) according to the method of Lohmann (1928). The precipitates were washed with 1 per cent. ammonia, transferred quantitatively to standard glass counting dishes, dried at 110°C., and the radio-activity estimated with a Geiger-Müller counter and scale of eight. Aliquots of diluted intestinal fluid were placed directly on the counting dishes, dried in a thin layer, and the ^{32}P content determined.

The mean error in the determination of radio-activity was ± 14 per cent. Corrections for self-absorption and resolving time were found to be unnecessary.

III. PROCEDURE AND RESULTS

Infested sheep, which had been fed on the laboratory stock ration, were each given intravenous or intra-abomasal injections of 10 ml. of a solution of sodium dihydrogen phosphate containing about 1 mc. of ^{32}P . After intervals of 2, 4, and 8 hr. animals were killed and samples of *Haemonchus contortus*, *Trichostrongylus* spp., and *Oesophagostomum columbianum* were collected from the abomasum, small intestine, and rectum respectively. Samples of gut mucosa from selected sites in the abomasum, small intestine, and rectum of the host were also taken. Samples of intestinal fluid were taken at a distance of 15-20 ft. from the pylorus. Some *Haemonchus* were found floating in the abomasal contents and others were picked off the abomasal mucosa. *Trichostrongylus* were removed from the intestinal mucosa with fine forceps under a dissecting microscope; *Oesophagostomum* were collected from the walls of the rectum and by washing the contents of the rectum through a sieve.

The parasites and the tissues of the host were washed repeatedly in 0.9 per cent. saline, the excess fluid removed with filter paper, and the wet weight determined. The weight ranged from 0.05 to 0.1 g. The material was then ashed and prepared for counting. Two samples were prepared for each ^{32}P determination and each experiment was carried out twice.

Similar trends were observed in duplicate experiments, but the values of the actual uptake of ^{32}P by the host tissues and by the parasites showed some variation. This variation was probably due to differences in such factors as intestinal motility, the rate of absorption, and the amount of food previously consumed by the sheep. The general physiological condition of the animals and the degree of parasitic infestation might also have affected the results obtained.

The values for the relative ^{32}P concentration as shown in the tables and graphs were calculated as the ratios of counts/min./g. wet wt. of tissue to dose (disintegrations/min.); the highest value recorded was taken as equivalent to 100 and the other values were appropriately adjusted.

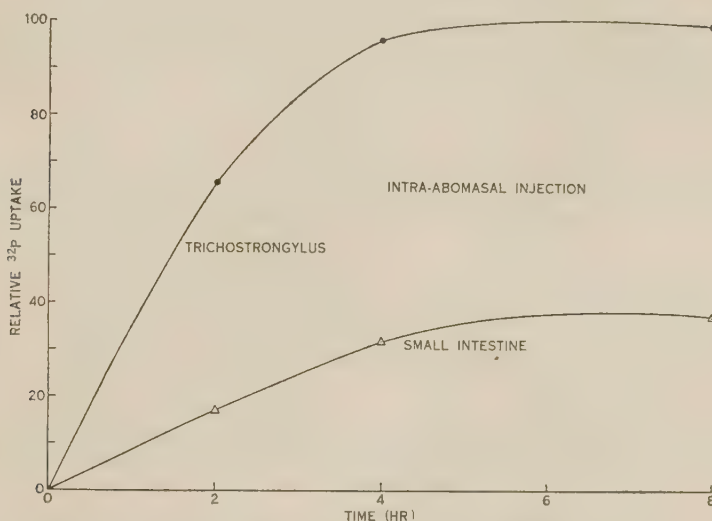


Fig. 1.—Relative amounts of radio-active phosphorus found in *Trichostrongylus* spp. and host gut tissues at different periods after dosing infested sheep intra-abomasally with sodium dihydrogen phosphate containing ^{32}P .

Figure 1 shows that *Trichostrongylus* taken from sheep that had been given an intra-abomasal injection absorbed approximately three times as much ^{32}P as the small intestine. The radio-activity of *Haemonchus* showed a considerable rise 8 hr. after the same type of dose whereas by this time the ^{32}P content of the abomasal tissue was decreasing (Table 1). The absorption of

TABLE 1
RELATIVE ^{32}P UPTAKE BY HOST TISSUES AND PARASITES AFTER INTRA-ABOMASAL INJECTION OF THE HOST

Sample	2 Hr.	4 Hr.	8 Hr.
Abomasum	1.5	25.4	21.5
<i>H. contortus</i>	17.7	18.2	42.0
Rectum	8.0	27.5	14.8
<i>O. columbianum</i>	2.3	4.5	No value available

The figures refer to the relative ^{32}P concentration, calculated as the ratio of counts/min./g. wet wt. to dose (disintegrations/min.).

^{32}P by *Oesophagostomum* following an intra-abomasal injection of the host could not be estimated, as there were not enough worms present in either of the sheep used for the 8-hr. experiments.

The results obtained after intravenous injections are shown in Table 2. The absorption of ^{32}P by *Trichostrongylus* followed a course parallel with the absorption by the small intestine; *Haemonchus* again appeared to be taking up an increasing amount of ^{32}P when the amount in the abomasal tissue was falling off. Variable results were obtained with *Oesophagostomum*; in one experiment the worms absorbed a much greater amount of ^{32}P than did the rectum, and in the other experiment, the ^{32}P uptake by the parasites and by the tissues of the rectum was of the same order.

TABLE 2
RELATIVE ^{32}P UPTAKE BY HOST TISSUES AND PARASITES AFTER INTRAVENOUS INJECTION OF THE HOST

Sample	2 Hr.	8 Hr.
Abomasum	68.4	46.9
<i>H. contortus</i>	49.7	74.0
Rectum	43.2	36.5
<i>O. columbianum</i>	5.3	79.0
Small intestine	53.0	78.8
<i>Trichostrongylus</i> spp.	59.7	85.2

The results refer to the relative ^{32}P concentration, calculated as the ratio of counts/min./g. wet wt. to dose (disintegrations/min.).

Figure 2 shows the relatively high ^{32}P content of the intestinal fluid of the host after an intra-abomasal injection compared with that obtained after an intravenous injection; no values were recorded for the latter at an 8-hr. period.

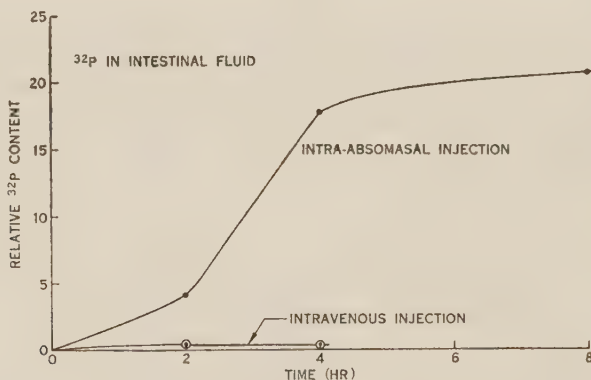


Fig. 2.—Relative amounts of radio-active phosphorus found in the intestinal fluid of sheep at different periods after dosing the sheep intra-abomasally and intravenously with sodium dihydrogen phosphate containing ^{32}P .

IV. DISCUSSION

As the fluid content of the host's intestine was found to be very low in radio-activity after the intravenous injection of labelled phosphate, the rapid

absorption of ^{32}P by *Trichostrongylus* spp. after such injection suggests that the parasites were feeding on the tissues of the host and not on the ingesta.

However, this method of feeding could not alone account for the great uptake of ^{32}P by the worms when labelled phosphate was given by injection into the abomasum. It seems, therefore, that the ^{32}P taken up by feeding on the tissues of the host may have been supplemented by phosphate absorbed through the cuticle.

Evidence that these nematodes are closely associated with the mucosa of the host gut has been discussed by Ackert and Whitlock (1940). Davey (1938), as a result of his *in vitro* experiments with nematode parasites of sheep, concluded that the forms with rudimentary buccal capsules probably feed on tissue elements at, or in, the mucosa. The results of the present investigation are in agreement with these suggestions.

Ransom (1911) classed *Haemonchus contortus* as a blood-sucking parasite; Broughton and Hardy (1935) observed the worms sucking blood from the wall of the abomasum. Haematological observations by one of us (P.M.S.), carried out over an extended period on sheep infested with *Haemonchus*, showed a definite correlation between decrease in red cell count and the degree of infestation. Ackert and Whitlock (1940) described these nematodes as being unattached to the mucous membrane but closely associated with it.

The relatively high absorption of ^{32}P by *Haemonchus contortus* when the sheep were given either intravenous or intra-abomasal injections of labelled phosphate suggested that the worms were feeding on the tissues of the host. Uptake through the cuticle did not appear to be important, as the relative amounts of ^{32}P in both the abomasum and its parasites were of the same order.

The feeding habits of nematodes are closely linked with the degree of the parasites' attachment to the tissues of the host. Very little information on the feeding habits of *Oesophagostomum columbianum* is available, but according to Ransom (1911), immature *Oesophagostomum* feed on the material in the nodules. From our observations, the adult worms appeared mainly to be lying on the walls or free in the contents of the rectum and not to be attached to the mucous membrane. Hoeppli (1927) emphasized the importance of examining the mode of attachment of parasitic nematodes to the host animal as soon as the host has been killed, as many parasites relinquish attachment shortly afterwards. However, in all our experiments the worms were collected immediately the sheep had been slaughtered.

In one series of experiments involving intravenous injections, *Oesophagostomum columbianum* showed a much higher ^{32}P uptake than did the tissues of the rectum, but when the experiments were repeated, the level of ^{32}P in the parasites was found to be similar to that in the tissue. These results, though variable, do show that absorption occurred quite rapidly. It is unlikely that appreciable amounts of labelled phosphate would have reached the contents of the rectum by 2-4 hr., or even 8 hr., after an intravenous injection, so that the high levels found in the parasites would appear to have been due to tissue

feeding. However, in the absence of any determinations on the radio-activity of the rectal contents, the results with regard to *Oesophagostomum* must be considered inconclusive.

In general it appears that the nematodes examined feed on the tissues of the host and not on the contents of the alimentary tract. Thus it seems unlikely that the parasites would take up an anthelmintic *per os* from the gut contents of sheep that have been dosed with the drug. Either the parasites absorb the anthelmintic via the cuticle or by the ingestion of the tissues of the host that contain the drug. With phenothiazine, the amount taken up by the intestinal mucosa of the host is small (Lazarus and Rogers 1950), so it seems probable that the major route of entry of the drug into the parasites would be via the cuticle.

V. ACKNOWLEDGMENT

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VI. REFERENCES

- ACKERT, J. E., and WHITLOCK, J. H. (1940).—"An Introduction to Nematology." (M. B. Chitwood: Babylon, N.Y.)
- BROUGHTON, I. B., and HARDY, W. T. (1935).—Ann. Rep. Texas Agric. Exp. Sta. No. 48: 238.
- DAVEY, D. G. (1938).—*Parasitology* 30: 278.
- HOEPLI, R. (1927).—*Arch. Schiffs-u. Tropen-Hyg.* 31: 207.
- LAZARUS, M., and ROGERS, W. P. (1950).—*Nature* 166: 647.
- LOHMANN, K. (1928).—*Biochem. Z.* 194: 304.
- MATHISON, G. C. (1909).—*Biochem. J.* 4: 233.
- RANSOM, B. H. (1911).—U.S. Dep. Agric. Bur. Anim. Industr. Bull. No. 127.
- READ, C. P. (1950).—*J. Parasit.* 36: 34.
- ROGERS, W. P., and LAZARUS, M. (1949).—*Parasitology* 39: 245.
- WRIGHT, W. H. (1950).—*J. Parasit.* 36: 176.

OBSERVATIONS ON THE PENETRATION OF THE SPERM INTO THE MAMMALIAN EGG

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Summary

A brief review is given of the literature, particularly that relating to the attempts made to effect the fertilization of the mammalian egg *in vitro*. It is considered that the evidence so far put forward for the fertilization *in vitro* of mammalian eggs is inconclusive.

Observations on eggs recovered at intervals after induced ovulation in mated rats indicate that sperm penetration of the zona pellucida occurs very rapidly and, generally, very soon after ovulation. As a rule, the sperm enters the vitellus immediately after passing through the zona, but quite often it remains for a period in the perivitelline space before entering the vitellus. The slit or potential hole the sperm makes in penetrating the zona persists and may be demonstrated at later stages.

Sperm entry into the vitellus has been observed *in vitro*; the process appears to be largely a function of the vitellus as the sperm is often motionless at the time.

When sperms were introduced into the fallopian tube of the rabbit *before* ovulation, most of the eggs subsequently recovered were fertilized. However, if the sperms were introduced shortly *after* ovulation the eggs rarely showed signs of penetration.

When sperms were introduced into the peri-ovarian sac of the rat shortly after ovulation, sperm penetration did not occur until four or more hours later, although sperms were regularly found about the eggs at two hours and later.

It appears therefore that the sperm must spend some time in the female tract before it is capable of penetrating the zona. These results and observations are discussed with the object of deriving a working hypothesis on the mechanism of sperm penetration through the zona pellucida.

I. INTRODUCTION

When it has reached the site of fertilization in the fallopian tube, the sperm of most species of mammals must yet pass three distinct barriers before it enters the egg to play its part in fertilization.

Much attention has been given to the nature of the first barrier, the cumulus oophorus, and the means whereby the sperm traverses it. Schenk (1878) noticed that the rabbit cumulus and that of the guinea pig were broken up when a suspension of sperms was added to it *in vitro*. It was not, however, until much later that Yamane (1930, 1935), Pincus (1930), and Pincus and Enzmann (1932, 1935) studied the reaction more closely and concluded that an agent resembling a proteolytic enzyme was involved. McClean and Rowlands (1942), Fekete

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and Duran-Reynals (1943), and Leonard and Kurzrok (1945) identified the enzyme as hyaluronidase, which has the effect of depolymerizing hyaluronic acid, the cement substance of the cumulus. The enzyme was shown to be carried by the sperm (Swyer 1946, 1947) and it was thought to function by denuding the egg as a preliminary to sperm entry. Leonard, Perlman, and Kurzrok (1947) and Austin (1948*a*, 1948*b*) pointed out that the action of the enzyme was probably limited to enabling the individual sperm to penetrate the intact cumulus.

There is little useful information available on the nature of the next two barriers, the zona pellucida and the surface of the vitellus, or on the manner in which the sperm traverses them.

The composition of the zona has been examined by several authors with varying results. Wallraff and Beckert (1939) detected polysaccharide in it, by the Bauer reaction. Leach (1947) considered the zona to be a mucoprotein, while Wislocki, Bunting, and Dempsey (1947) and Leblond (1950) described it as a mucopolysaccharide. Harter (1948), on the other hand, stated that glycoprotein could be demonstrated in the zona.

Some of the physical properties of the zona have also been investigated. Mayer (1842) observed that the zona was dissolved in a solution of potassium hydroxide of unstated concentration, and concluded that it was a single solid membrane, and not two thin membranes separated by a layer of protein. The removal of the zona from rabbit eggs after treatment with osmic acid and Muller's fluid (which contains potassium dichromate) was noted by Van Benedin (1875). Lams (1913) remarked that the guinea pig zona was occasionally dissolved by Zenker's and Hermann's fluids, both of which contain acetic acid. Huber (1915) observed the absence of the zona in many of the rat eggs fixed in Carnoy's fluid, which also contains acetic acid. More recently, Hall (1935), following this lead, studied the effect of pH on the mouse zona and found that it was dissolved readily at pH 3.7, more slowly at less acid reactions up to pH 5.4. Harter (1948) suggested that the lowered pH produced by the metabolism of the sperm in the immediate vicinity of the head might assist penetration of the egg by its solvent action on the zona.

The claims of some early workers, notably Barry (1840), that the continuity of the zona was broken by an orifice or "cleft," by means of which it was supposed the sperm might enter, have not been supported. In the mature ovarian egg, the zona appears as a thick, refractile membrane and, in fixed material, fine radial canals containing processes from the follicle cells may be seen crossing it, as has been described by Heape (1886), Nagel (1888), Sobotta (1895), Fischer (1905), and many other workers subsequently. The processes from the follicle cells may also be seen in living ovarian eggs (Austin and Smiles 1948). The zona of the tubal egg, however, appears to be homogeneous (Lams 1913) or may show fine radial striations (Heape 1886) or a faint, concentric layering (Corner 1928). There is, therefore, no sound evidence of any form of selected path or easy way for the passage of the sperm through the zona.

Concerning the nature of the third barrier, the surface of the vitellus, there has also been some difference of opinion. The earlier workers, from Barry (1839) to Lams (1913), were almost unanimous in referring to a vitelline membrane as a structure which surrounded the vitellus in both fertilized and unfertilized mammalian eggs. There is, however, no good reason for supposing the existence of an actual membrane around the vitellus, analogous to that in a bird's egg (Corner 1928). Nevertheless, it has long been recognized that the vitelline surface may present a distinct obstacle to sperm penetration in that it becomes modified in some way after the penetration of the first sperm so as normally to exclude the entrance of later sperms. The nature of this change is still a matter for speculation. Pincus and Enzmann (1932) and Gilchrist and Pincus (1932) showed that the vitellus in the rabbit and rat eggs suffers a shrinkage after sperm penetration, but, although the appearance of this change may suggest it, there is no evidence for the formation of a fertilization membrane as described in certain invertebrate eggs.

Undoubtedly the study of the mechanisms involved in the penetration of the zona and vitelline surface would be greatly facilitated if the fertilization of mammalian eggs could be achieved *in vitro*. During the past century, several workers have claimed success in this procedure, but it is difficult to decide whether these claims were properly founded. The main difficulty lies in distinguishing between effects resulting from sperm penetration and those due to parthenogenetic activation. There is also a danger that sperms introduced accidentally during the sectioning of the eggs may be mistaken for sperms that have entered the eggs in the normal way.

Schenk (1878) treated the ovarian eggs of rabbits and guinea pigs with sperms *in vitro* and noted the formation of a polar body and, following culture, the division of the egg.

Onanoff (1893), in a posthumous communication in which only conclusions were published, made a remarkable series of claims. He stated that rabbit and guinea pig eggs, taken from the uterus, could be fertilized *in vitro*, and that their development would proceed to the 8-cell stage. Eggs fertilized *in vitro* and transferred to the peritoneal cavity of males or females of either species would develop into embryos of the primitive streak stage.

Long (1912) described the break-up of the cumulus and the formation of the second polar body in rat eggs treated with sperms *in vitro*.

Frommolt (1934) mentioned the shrinkage of the vitellus as the sole criterion of fertilization in the rabbit eggs to which he had added sperms *in vitro*.

Krasovskaja (1934, 1935a, 1935b) claimed the fertilization *in vitro* of rabbit eggs with the sperms not only of the rabbit, but also the rat. The evidence included the abstriction of the second polar body, the formation of pronuclei and the division of the egg when cultured. She did not apparently observe the presence of any sperms within the eggs. Pincus (1936) remarks that the nuclear configurations shown by Krasovskaja can occur when eggs are cultured *in vitro* without the addition of sperms.

Yamane (1935), who studied the dispersion of the follicle cell mass by a suspension of sperms *in vitro*, considered that several of the eggs so treated had sperms within the vitellus. Two of the eggs are illustrated in Yamane's paper and as judged from these the evidence is not convincing. There appear to be several sperm heads in, or partly in, the vitellus, and these show no change towards pronucleus formation. The whole appearance strongly suggests that the heads in reality overlie the section of the vitellus and that they were carried there in the cutting of the section.

The most extensive experiments on the problem of fertilization *in vitro* have been carried out by Pincus (1930, 1939) and Pincus and Enzmann (1934, 1935). These authors added ejaculated and epididymal sperms to rabbit eggs *in vitro*. Both ovarian and tubal eggs were used and the fertilization of many of these was claimed. The evidence submitted included the shrinkage of the vitellus, the extrusion of the second polar body, the presence of sperms in the perivitelline space and vitellus as seen in histological sections, the formation of two pronuclei, the segmentation of the eggs, and the birth of young. These authors also made the point that, if sperm penetration is to occur, there must be a relatively high concentration of sperms (25,000 per cu. mm. or more) about the eggs. At lower concentrations the follicle cell mass surrounding the eggs was not completely removed and fertilization did not take place.

In evaluating this evidence the following points should be noted. Gilchrist and Pincus (1932) showed that, in rat eggs, shrinkage of the vitellus could be induced by incubating the eggs with a suspension of dead sperms. Pincus and Enzmann (1936), Pincus (1939), and Pincus and Shapiro (1940) reported that, by subjecting rabbit eggs *in vitro* to supra- or subnormal temperatures, hyper- or hypotonic solutions, butyric acid solution, or culturing in a moist chamber, varying degrees of activation were induced. They have recorded that the extrusion of the second polar body, the formation of two pronuclei, the segmentation of the egg, and even the birth of young could all be induced in the absence of sperms. More recently Thibault (1947*a*, 1947*b*, 1948) has studied the artificial activation of the rabbit egg by cold and noted that the egg may subsequently show the formation of a single, diploid nucleus or two nuclei closely resembling normal pronuclei. He also stated that the first polar body, following cold treatment of the egg, may divide so that the egg appears to have two normal polar bodies. Furthermore, it is known that even under normal condition *in vivo* the unfertilized eggs of the rat, mouse, and ferret may fragment in such a manner as closely to resemble segmented fertilized eggs (Austin 1949; Chang 1950*a*).

With regard to the high concentrations of sperms required and the removal of the follicle cell mass before fertilization, it has been observed by other authors that these conditions do not apply to normal fertilization *in vivo*. Thus, remarkably few sperms are to be found *in vivo* in the vicinity of the recently fertilized egg (Tafari 1889; Sobotta 1895; Sobotta and Burckhard 1910; Hammond 1925; Hammond and Walton 1934; Austin 1948*b*; Blandau and Odor 1949). More-

over, removal of the follicle cell mass is not necessary for fertilization (Lewis and Wright 1935; Leonard, Perlman, and Kurzrok 1947; Austin 1948*a*, 1948*b*; Blandau and Odor 1949).

The fertilization *in vitro* of human ovarian eggs, which were also matured *in vitro*, is claimed by Rock and Menkin (1944) and Menkin and Rock (1948). Their evidence consists of an identification of sperm heads within the bounds of the zona in histological sections, and the segmentation of some of the eggs during subsequent culture.

It is clear that there is only one unequivocal sign that permits a distinction to be drawn between non-specific activation and fertilization, and that is the presence of the sperm within the egg. This evidence, however, is difficult to establish positively in histological material where any small basophilic object may be interpreted as a sperm head, and where true sperm heads may have been introduced into the egg section accidentally.

It cannot be said that any useful information on the mechanism of sperm penetration of the zona and vitellus, or on the conditions influencing this process, has come from the reports on *in vitro* fertilization reviewed above. Recently, however, attempts have been made to define certain of the conditions required for penetration (Moricard and Bossu 1949*a*, 1949*b*; Moricard 1949, 1950). These authors stated that if eggs, still enclosed in pieces of fallopian tube, were treated with sperms under a layer of "vaseline oil," and incubated for periods up to seven hours, sperm heads could be identified in the zona, perivitelline space, and vitellus. No penetration was observed when the experiment was carried out under aerobic conditions and in the absence of fallopian tube. The authors had observed that a reducing potential existed within the normal fallopian tube and conclude that this is an important requirement for sperm penetration.

The observations now to be described are recorded in the hope that they may throw some light on the mechanisms involved in the passage of the sperm through the zona and vitelline surface.

II. METHODS

Observations were made with adult and 40-55-day old immature rats, and with adult rabbits.

Ovulation was induced in the immature rats by injection of 20 I.U. of pregnant mare's serum (B.D.H. "Serogan") followed 48-56 hours later by 20 I.U. of chorionic gonadotrophin (B.D.H. "Gonan"), according to the procedure described by Rowlands (1944). In the rabbits, ovulation was induced by injecting 50 I.U. chorionic gonadotrophin intravenously.

For the surgical work on both the rats and rabbits ether anaesthesia was found most satisfactory, accompanied in the rabbits by atropine premedication.

The eggs were examined with a phase-contrast microscope, by the method described by Austin and Smiles (1948).

III. OBSERVATIONS

(a) Time Relations of Sperm Penetration after Mating in Immature Rats

For the assessment of time relations, the immature rat was selected because of the reliability with which ovulation may be induced in this animal, and the high suitability of the eggs for phase-contrast microscopy.

In the initial experiments, which were designed to show the time of ovulation, rats were killed at hourly intervals from 11 to 14 hours after the injection of chorionic gonadotrophin. The fallopian tubes were removed and examined by dissection under normal saline. The presence of eggs in the tubes was taken as the criterion of the occurrence of ovulation. The results (Table 1) show that ovulation occurs mostly 12-13 hours after the injection of chorionic gonadotrophin.

TABLE 1
TIME OF INDUCED OVULATION IN IMMATURE RATS

Time Killed After Injection of Chorionic Gonadotrophin (hr.)	Total Numbers of Rats Used	Rats in which Ovulation had Occurred	
		No.	Percentage
11	25	3	12
12	29	18	62
13	23	19	83
14	21	20	95

In the next series of tests, the immature female rats were placed with adult males immediately after the injection of chorionic gonadotrophin and examined for the presence of the copulation plug 10-16 hours later. Mated rats were killed at intervals ranging from 11 to 32 hours after the injection. The eggs were examined for evidence of sperm penetration and the results are shown in Table 2.

All the rats that mated and provided eggs had sperms in at least a proportion of the eggs. Data were obtained from 10 rats at each of the times selected, except at 11 hours after chorionic gonadotrophin when the frequency of ovulation is very low. At 11 hours a little over one-third of the eggs contained sperms in the perivitelline space and in the vitellus whereas at later times from 61 to 87 per cent. of eggs showed sperms in these locations. At 12 hours most of the eggs recovered had sperms within the vitellus.

In a total of 54 eggs there were sperms in the perivitelline space but not in the vitellus; 42 of these eggs came from rats killed 12-18 hours after chorionic gonadotrophin and only seven from the rats killed at 20-32 hours. Pronuclei were seen in the eggs from seven of the 10 rats killed as early as 12 hours after chorionic gonadotrophin.

Supernumerary sperms (i.e. sperms in the perivitelline space of eggs that have also a sperm in the vitellus) were frequently seen. The largest number of supernumerary sperms observed was 23. Plate 1, Figure 4, shows this egg, but only about 20 sperms can be identified at the focal plane selected. These sperms are all in the perivitelline space although in the photograph they appear to be within the vitellus.

(b) *Sperm Penetration after Injection of Sperms into the Peri-ovarian Sac of Immature Rats*

Ovulation was induced in groups of immature rats by the method described. At 16-17 hours after the injection of chorionic gonadotrophin (i.e. about 3-5 hours after ovulation) a small volume (0.001-0.01 ml.) of a suspension of epididymal sperms was injected with a fine needle into the peri-ovarian sac on each side. Runner (1947) obtained normal pregnancy in the mouse by introducing sperms in this manner. In the present investigation the sperm suspension was made with an isotonic phosphate buffer solution at pH 7.2 and kept under paraffin. The rats were killed at intervals from 2 to 8 hours and at 24 hours after the injection of sperms, and the eggs were examined for evidence of penetration.

TABLE 2
DISTRIBUTION OF SPERMS IN THE EGGS FROM MATED RATS KILLED AT INTERVALS
AFTER THE INJECTION OF CHORIONIC GONADOTROPHIN

	Hours After Injection when Killed						
	11	12	14	16-18	20	24	28-32
Number of eggs having sperms in peri-vitelline space but not in vitellus	5	10	16	16	5	1	1
Sperms in vitellus	8	115	92	140	168	185	167
Total number of eggs containing sperms	13	125	108	156	173	186	168
Total number of eggs examined	35	157	175	254	201	243	194
Number of rats providing eggs	2	10	10	10	10	10	10

The results (Table 3) show that none of the eggs obtained before 4 hours after the injection of sperms showed any penetration. At 4 hours only one egg in a total of 301 had a sperm within. After 4 hours eggs quite frequently contained sperms, the highest proportion being observed in the rats killed at 24 hours. These provided 203 eggs, of which 34 contained sperms. In all rats, including those killed at 2, 3, and 4 hours after the injection of sperms, numerous sperms were observed in the tubes and surrounding the eggs. When the larger volumes of sperm suspension were used, as in groups 1, 2, 8, and 11 (Table 3) all eggs were free of follicle cells when recovered.

(c) *Sperm Penetration after Introduction of Sperm Suspensions into the Fallopian Tubes of Rabbits*

Sperm suspensions were introduced into the fallopian tubes of rabbits under ether anaesthesia. The sperms were obtained from the epididymis of adult male rabbits and used either undiluted or diluted with a buffered saline.

Introduction was effected by means of a fine glass tube attached to a micro-meter syringe. Two groups of rabbits were used: in the first group (13 rabbits), sperms were introduced 12-14 hours after the injection of chorionic

TABLE 3
NUMBER OF EGGS SHOWING SPERM PENETRATION EXPRESSED AS FRACTIONS OF
TOTAL EGGS RECOVERED FROM RATS KILLED AT INTERVALS AFTER INJECTION
OF SPERM SUSPENSION INTO PERI-OVARIAN SAC

Expt. No.	Time After Injection of Sperms (hr.)							
	2	3	4	5	6	7	8	24
1	0/15		0/10					
2	0/15		0/14		0/3 5/13			
3								7/12 0/18 4/10 0/1 4/7
4			0/31	0/25	0/8 0/19	2/16 10/25		
5								7/23 0/33 3/15 6/28
6		0/38	0/11	0/17	5/21	1/11		
7				0/47	0/21	0/28	1/26 3/36 4/35	
8		0/16	0/61	0/9	1/13	4/13 0/21		
9	0/14	0/7	0/35	1/47	0/7			
10	0/20	0/16	0/9	2/15	0/7			
11	0/65	0/13	0/29	0/23	0/5	1/43	2/10	
12	0/6	0/41	1/11	0/14		3/38	1/9	
13	0/40 0/8	0/18	0/61	0/10	0/17			2/21 1/15 0/3 4/17
14	0/35	0/9 0/20	0/29	0/52 1/18	2/25			
Total	0/218	0/178	1/301	4/277	13/159	21/195	11/116	34/203
Percentage	0	0	0.3	1.4	8.1	10.8	9.5	16.7

gonadotrophin (about 2-4 hours *after* ovulation); in the second group (6 rabbits), sperms were introduced 3-5 hours after the injection of chorionic gonadotrophin (about 5-7 hours *before* ovulation). The rabbits were killed, mostly more than 30 hours after ovulation, and the eggs examined for evidence of penetration and fertilization. The results (Table 4) show that where the

sperms were introduced after ovulation only two eggs in a total of 63 contained sperms. On the other hand (Table 5), when sperms were introduced before ovulation, sperm penetration occurred in 19 of the 25 eggs recovered.

TABLE 4
RESULTS OBTAINED FROM INTRODUCING SPERM SUSPENSION INTO THE FALLOPIAN TUBES OF RABBITS SHORTLY AFTER OVULATION

Rabbit No.	Sperm Suspension Introduced		Hours After Ovulation when Killed	Results
	Dilution	Volume		
1	Undiluted	0.03 ml.	7½	7 × 1-cell eggs. No sperms within
2	Undiluted	0.03 ml.	6½	3 × 1-cell eggs. No sperms within
3	Undiluted	0.03 ml.	32	7 × 1-cell eggs. No sperms within. Sperms numerous in "albumen"
4	Undiluted	0.03 ml.	51	5 × 1-cell eggs. No sperms within. Sperms numerous in "albumen"
5	1 : 10	0.03 ml.	32	4 × 1-cell eggs. No sperms within. Sperms numerous in "albumen"
6	1 : 10	0.03 ml.	33	5 × 1-cell cells. No sperms within. Sperms numerous in "albumen"
7	Undiluted	0.005 ml.	32	3 × 1-cell eggs. No sperms within
8	Undiluted	0.0025 ml.	33	4 × 1-cell eggs. No sperms within. 1 × 16 cell egg, with 1 sperm traversing the zona
9	1 : 8	0.002 ml.	32	3 × 1-cell eggs. No sperms within. 1 × 2 cell egg, with 1 sperm in zona and 4 in perivitelline space
10	1 : 8	0.001 ml.	33	6 × 1-cell eggs, 1 egg fragmented. No sperms within
11	Undiluted	0.001 ml.	31	4 × 1-cell eggs. No sperms within. Some sperms in "albumen"
12	1 : 10	0.003 ml.	31	4 × 1-cell eggs. No sperms within. Some sperms in "albumen"
13	1 : 10	0.003 ml.	32	5 × 1-cell eggs. No sperms within. Some sperms in "albumen"

(d) *General Observations on Sperm Penetration in vivo*

Examination of eggs recovered from mated rats soon after ovulation has shown that a sperm may occupy any one of five positions in the egg. It may lie (a) totally within the vitellus, (b) with a variable part of the mid-piece or tail still in the perivitelline space, or (c) with the tail still projecting through the zona into the surrounding medium. It may also lie (d) totally within the perivitelline space or (e) with its tail still projecting through the zona. By far the commonest form observed is (a), but (d) is quite frequently seen. The other forms are somewhat rare. An egg showing form (c) is illustrated in Plate 1, Figure 1.

Eggs examined later on, however, towards the time of the first segmentation, very rarely have sperms in any position except totally within the vitellus,

for the fertilizing sperm, or totally within the perivitelline space, for any supernumerary sperms.

When eggs having sperms traversing the zona are examined from above the point of penetration, the sperm tail is seen to project through what appears to be an elliptical hole in the zona. Often, as a result of the pressure exerted by the overlying cover-slip, some of the contents of the egg exude through the hole past the sperm tail. A similar slit or potential hole can usually be recognized in eggs that contain sperms even though the tails do not project through. It has never been seen, however, in eggs that do not contain sperms. If more than one sperm lies within an egg, it is usually possible to discern more than one slit in the zona, and often as many as there are sperms within. The number of slits has never exceeded the number of sperms. The appearance of the slit is shown in Plate 1, Figure 2. When this egg was rolled under the cover-slip so that the slit came to the free surface at the side of the egg, the cytoplasm of the vitellus immediately exuded, as may be seen in Plate 1, Figure 3. These signs of sperm entry have been seen in eggs recovered just before segmentation and may well persist for much longer.

TABLE 5
RESULTS OBTAINED FROM INTRODUCING SPERM SUSPENSION INTO THE FALLOPIAN
TUBES OF RABBITS ABOUT SIX HOURS BEFORE OVULATION

Rabbit No.	Sperm Suspension Introduced		Hours After Ovulation when Killed	Results
	Dilution	Volume		
14	Undiluted	0.001 ml.	42	1 × 1-cell egg. No sperms within
15	Undiluted	0.001 ml.	42	3 × 1-cell eggs. No sperms within. 1 × 16-cell egg, sperms seen with- in
16	1 : 10	0.001 ml.	42	2 × 16-cell eggs. Sperms seen within
17	1 : 10	0.001 ml.	42	5 × 16-cell eggs. Sperms seen in all eggs
18	1 : 10	0.001 ml.	42	1 × 1-cell egg. 6 × 16-cell eggs, sperms seen within
19	1 : 10	0.001 ml.	42	1 × 1-cell egg. 5 × 16-cell eggs, sperms seen within

(e) *Observations made on Eggs in vitro*

Eggs have been obtained within three or four hours of ovulation from both rats and rabbits, and have then been subjected to certain tests. In the main these have involved suspending the eggs in a variety of media and adding the appropriate sperms. With adequate sperm concentrations the follicle cell mass about both species of eggs is rapidly broken up. The rat egg is easily denuded but the rabbit egg retains a layer of follicle cells (the "corona"), which is only removed by very high concentrations of sperms. When rabbit eggs are treated with sperm suspensions *in vitro* the sperms adhere to the zona by the anterior part of the head, so that, with high concentrations, the entire

surface of the egg becomes covered with firmly adherent sperms. This does not happen to the rat egg; only occasionally are sperms found attached to the zona and even then the attachment is very weak and the sperms are readily brushed off. No concentration of sperms has been found to have any observable effect upon the zona in either species.

Penetration of the sperm through the zona was never observed. Unsuccessful tests included the removal of rat eggs from the fallopian tube under paraffin so that the eggs were suspended only in tubal fluid. Epididymal sperms and sperms obtained from the uteri of mated rats were added to these eggs, and the preparations were incubated at 37°C. for up to 7 hours. No penetration occurred.

In confirmation of Hall's (1935) statement, the rat zona has been found to pass into solution in weakly acid media; readily at a pH less than 5, more slowly and uncertainly at a pH between 5.5 and 6.5.

With both rat and rabbit eggs, the zona is dissolved in solutions of some reducing agents at pH 7-8, as well as at acid reactions. Thus 0.01M glutathione and 0.01M cysteine in Tyrode solution caused rapid dissolution of the zona whereas 0.01M ascorbic acid was almost without effect.

Penetration by sperms through the surface of the vitellus has now been observed *in vitro* on several occasions. Eggs obtained from mated, immature rats within a few hours after ovulation frequently have sperms in the perivitelline space but no sperm, as yet, in the vitellus (as indicated above in (a) and Table 2). A number of these eggs have been kept under observation at 30-37°C. for several hours, and in most of them the penetration of the head into the vitellus was seen. Usually this occurred with the head lying flat upon the surface of the vitellus but sometimes the pointed extremity of the hook-shaped head preceded the rest. Penetration generally happened within an hour from the commencement of observations, but in one case it took place near the end of the third hour. The process of penetration involved simply the gradual sinking of the sperm head into the substance of the vitellus. Sometimes the sperm continued to show some motility during its entry into the vitellus, but more often it was quite motionless. First the mid-piece and then the tail slowly followed the head into the vitellus.

IV. DISCUSSION

In this paper observations are described which relate to the penetration of the sperm into the egg of the rat and rabbit, attention being directed particularly to the passage of sperms through the zona pellucida and the surface of the vitellus.

Examination of eggs recovered at intervals after normal mating in rats provided data for the following conclusions:

(a) The penetration of the sperm into the egg is a very rapid process. It probably takes no more than a few minutes at most for the head of the sperm to pass through the zona, judging by the fact that among over 1,200 eggs

examined none showed a sperm head in the thickness of the zona. The frequency with which supernumerary sperms are seen and the number (as many as 23) in one egg show that the sperm has no difficulty in traversing the zona.

(b) Penetration into the egg generally occurs very shortly after the arrival of the eggs in the fallopian tube and in some instances may occur within a few minutes. This is shown by the frequent occurrence of well-formed pronuclei in eggs recovered at 12 hours after the ovulating injection, i.e. probably well within an hour of ovulation.

(c) Penetration into the egg can occur in either one or two stages: the sperm may pass straight through the zona and into the vitellus, or it may remain for a variable period in the perivitelline space before entering the vitellus. Penetration without pause appears to be the more general occurrence. The two-stage process, however, is not uncommon; between 10 and 20 per cent. of penetrated eggs, recovered within 5 or 6 hours of ovulation, had sperms in the perivitelline space but not in the vitellus. It seems clear that these sperms would later have entered the vitellus, as very few eggs recovered 8 or more hours after ovulation have sperms only in the perivitelline space.

(d) When the sperm passes through the zona of the rat egg, it leaves a slit or potential hole, which can readily be demonstrated. The slit has been seen as late as the stage of the first segmentation and may well persist for longer than this. It cannot constitute a serious point of weakness in the zona for the latter is a strong elastic membrane and the contents of the egg can only be made to flow through the slit when the egg is firmly compressed under a cover-slip. The sperm apparently penetrates the zona at any point on the surface; when several sperms have entered a single egg the slits are seen often at widely different parts of the zona.

The entry of the sperm head into the vitellus occurs readily *in vitro* and has been observed on several occasions. The head sinks into the vitellus and, within a few minutes, begins to undergo the series of changes leading to the formation of the male pronucleus (Austin 1951). The rest of the sperm is gradually taken into the vitellus in the same manner. During these events as observed *in vitro*, the sperm is often quite motionless. Penetration into the vitellus thus appears to be a function of the vitellus itself. However, nothing has been observed to suggest that the mid-piece or tail are ever taken into the vitellus before the head. There must therefore be some property of the head that results in its being absorbed into the vitellus first.

Penetration of the sperm through the zona pellucida is clearly a process of a different nature. Although the zona presents little or no obstacle to the sperm *in vivo* under normal circumstances, penetration *in vitro* has not been observed in any of the preparations, even when the use of artificial media was avoided. Furthermore, the evidence obtained from the animal experiments showed that when sperms were introduced into the female tract, in both rats and rabbits, they were not able immediately to penetrate the zona. In the rabbits, 19 out of 25 eggs were penetrated when sperms were introduced into the fallopian tubes before ovulation, but only two eggs out of 63 were

penetrated when the sperms were put in 2-4 hours after ovulation. Presumably the eggs become unfertilizable before the sperms acquired the capacity for penetration. Hammond (1934) considered that the eggs of the rabbit remained fertilizable for not more than 6 hours after ovulation.

The rats provided a slightly different picture, probably because rat eggs remain fertilizable for about 12 hours (Blandau and Jordan 1941; Soderwell and Blandau 1941). When sperms were introduced into the peri-ovarian sac 3-5 hours after ovulation no penetrated eggs were found until 4 hours after the operation. At 4 hours and later, 84 eggs out of a total of 1251 were found to contain sperms. This is not a high proportion, but it should be remembered that the actual times were close to the limit of the fertilizable life of the rat egg. In the rats killed 2 and 3 hours after operation, sperms were always seen among the eggs, often in quite large numbers, and yet, as just stated, none of these eggs was penetrated. Here again there seems to be a need for the sperms to spend some time, apparently a few hours, in the female tract before they can penetrate the zona.

The following further conclusions seem to be justified:

(a) Sperms freshly obtained from the epididymis are incapable of penetrating the zona immediately, even *in vivo* and under conditions that must closely resemble the normal.

(b) The sperms must remain within the female tract for a period before they are able to penetrate the eggs.

(c) The sperms need not pass through the uterus; it is sufficient that they reside for a period in the peri-ovarian sac or in the fallopian tube.

(d) Although eggs are normally penetrated whilst surrounded by an apparently intact cumulus, they may still be penetrated even though denuded some hours previously. This is indicated by the presence of sperms in eggs recovered 6, 7, and 8 hours after the injection of relatively large amounts of sperm suspension into the peri-ovarian sac (Expt. Nos. 1, 2, 8, and 11 in Table 3). All the eggs recovered at earlier hours in these groups were denuded. Evidently the physical presence of the cumulus is not required by the sperm when it begins to make its way through the zona, and the egg, in the rat at least, does not, as Chang and Pincus (1951) suggest, become unfertilizable when it is denuded.

It is of interest to note here that in the rat and rabbit, under normal circumstances, mating takes place much longer before ovulation than the time taken for the sperms to reach the site of fertilization. Thus in the rabbit, ovulation occurs 9-10 hours after mating (Barry 1839; Heape 1905) and the sperms reach the ovarian end of the fallopian tube in 4 hours (Heape 1905) or probably less. The corresponding times for the rat are 8-11 hours (Blandau, Boling, and Young 1939), and 15-30 minutes respectively (Blandau and Money 1944).

The principal conclusion arising from the work described in this paper is that, at least in the rat and rabbit, the sperm must undergo some form of preparation or capacitation before it can penetrate the zona, and that this process is normally effected in the fallopian tube.

Such a conclusion is plainly in conflict with the numerous claims on the attainment of *in vitro* fertilization reviewed in the introductory section of this paper. These claims, however, were based on evidence that must be considered inconclusive. It therefore seems highly probable that the fertilization of mammalian eggs *in vitro* has not yet been achieved. Consistent with this is the fact that some other workers have also reported negative results (Umbaugh 1949; Chang 1950*b*; Moricard 1950).

The work of Moricard and Bossu (1949*a*, 1949*b*) belongs to a different category, for, in spite of their use of the term *in vitro*, their experiments involved sperm penetration in the isolated tube and not strictly *in vitro*. These authors observed sperm penetration only after the sperms had been incubated in pieces of fallopian tube for several hours, and their findings are therefore also consistent with the observations recorded in the present paper.

The process whereby the sperm makes its way through the zona has yet to be determined. Harter's (1948) suggestion that an acid reaction about the sperm head is the active agent is unattractive for two reasons. Firstly, if it were true, one would expect the dissolution of the entire zona when the egg is incubated in the presence of a high concentration of sperms, and this has not been observed. Secondly, Harter's suggestion could not apply to the penetration of the rabbit zona, which is not removed by treatment with acid media down to pH 3 (Braden, personal communication 1950). In some respects there is a better case to be made out for a reducing potential as the active agent, because this, as noted in the text, is effective on both the rat and rabbit zona. However, this theory is also objectionable on the grounds that, if it were true, a high concentration of sperms should remove the entire zona.

In general, the data obtained seem to favour the possibility that a specific agent, in the nature of a mucolytic enzyme, is carried by the sperm and assists its penetration by digesting a path through the zona. It is further suggested that an inhibitor normally accompanies the sperm and must be removed before the agent can act upon the zona. The removal of the inhibitor by absorption through the mucosa of the tube may well be a slow process and this would account for the relatively long sojourn in the female tract required by the sperm after injection into the tube or peri-ovarian sac. It is quite possible that in the normal circumstances after mating the time required to be spent by the sperm in the female tract would be less, because the supposed inhibitor could be more effectively removed when the sperm must traverse the whole length of uterus and tube.

This hypothesis is, of course, highly speculative. More information is required, particularly on the nature of the processes involved in the capacitation of the sperm and on the composition and properties of the zona. These problems are now under investigation in this laboratory.

V. REFERENCES

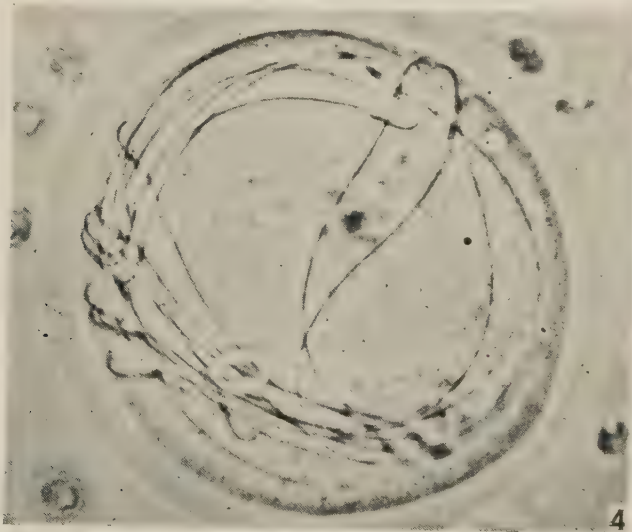
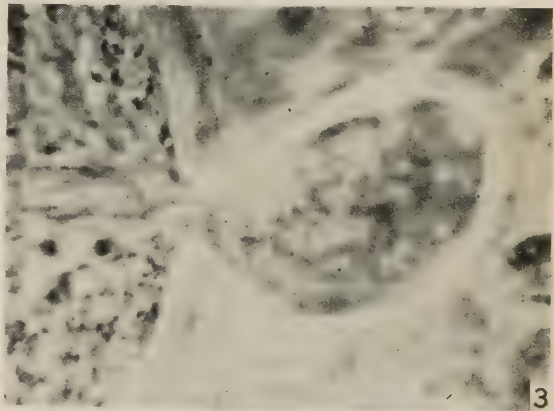
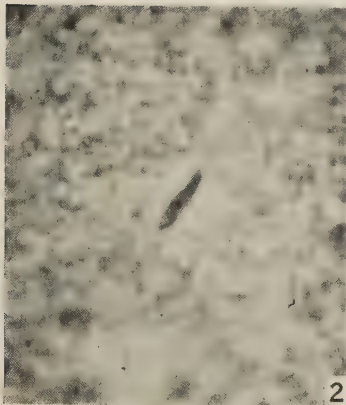
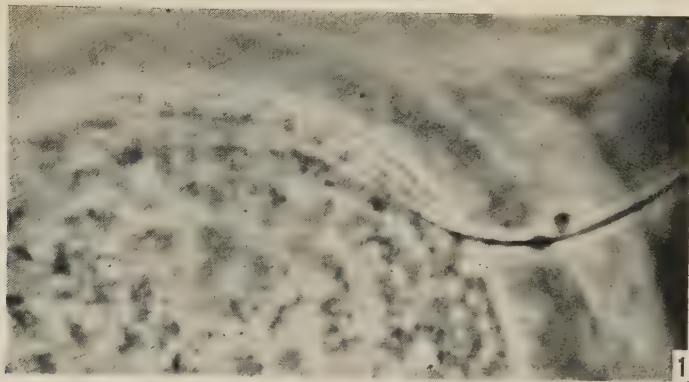
- AUSTIN, C. R. (1948*a*).—*Nature* **162**: 63.
 AUSTIN, C. R. (1948*b*).—*Nature* **162**: 534.
 AUSTIN, C. R. (1949).—*J. Endocrinol.* **6**: 104.
 AUSTIN, C. R. (1951).—*J. R. Micr. Soc.* (in press).
 AUSTIN, C. R., and SMILES, J. (1948).—*J. R. Micr. Soc.* **68**: 13.
 BARRY, M. (1839).—*Philos. Trans.* Pt. 2: 307.
 BARRY, M. (1840).—*Philos. Trans.* Pt. 2: 529.
 BLANDAU, R. J., BOLING, J. L., and YOUNG, W. C. (1939).—*Anat. Rec.* **75**: 74 (Suppl.).
 BLANDAU, R. J., and JORDAN, E. S. (1941).—*Amer. J. Anat.* **68**: 275.
 BLANDAU, R. J., and MONEY, W. L. (1944).—*Anat. Rec.* **90**: 255.
 BLANDAU, R. J., and ODOR, D. L. (1949).—*Anat. Rec.* **103**: 93.
 CHANG, M. C. (1950*a*).—*Anat. Rec.* **108**: 31.
 CHANG, M. C. (1950*b*).—*Fertility and Sterility* (in press). (Cited by Chang and Pincus 1951.)
 CHANG, M. C., and PINCUS, G. (1951).—*Physiol. Rev.* **31**: 1.
 CORNER, G. W. (1928).—Section XXXII of "Special Cytology," Vol. 2, Ed. E. V. Cowdry. (Paul B. Hoeber Inc.: New York.)
 FEKETE, E., and DURAN-REYNALS, F. (1943).—*Proc. Soc. Exp. Biol., N.Y.* **52**: 119.
 FISCHER, A. (1905).—*Anat. Hefte.* **29**: 555.
 FROMMOLT, G. (1934).—*Zbl. Gynäk.* **58**: 7.
 GILCHRIST, F., and PINCUS, G. (1932).—*Anat. Rec.* **54**: 275.
 HALL, B. V. (1935).—*Proc. Soc. Exp. Biol. N.Y.* **32**: 747.
 HAMMOND, J. (1925).—"Reproduction in the Rabbit." (Oliver and Boyd: Edinburgh.)
 HAMMOND, J. (1934).—*J. Exp. Biol.* **11**: 140.
 HAMMOND, J., and WALTON, A. (1934).—*J. Exp. Biol.* **11**: 307.
 HARTER, B. T. (1948).—*Anat. Rec.* **102**: 349.
 HEAPE, W. (1886).—*Quart. J. Micr. Sci.* **26**: 157.
 HEAPE, W. (1905).—*Proc. Roy. Soc. B* **76**: 260.
 HUBER, G. C. (1915).—*J. Morph.* **26**: 247.
 KRASOVSKAJA, O. V. (1934).—*Russk. Arkh. Anat.* **13**: 415. (Cited by Menkin and Rock 1948.)
 KRASOVSKAJA, O. V. (1935*a*).—*Acta Zool.* **16**: 449.
 KRASOVSKAJA, O. V. (1935*b*).—*Biol. J.* **4**: 262. (Cited by Pincus 1936.)
 LAMS, H. (1913).—*Arch. Biol.* **28**: 229.
 LEACH, E. H. (1947).—*Stain Tech.* **22**: 73.
 LEBLOND, C. P. (1950).—*Amer. J. Anat.* **86**: 1.
 LEONARD, S. L., and KURZROK, R. (1945).—*Endocrinology* **37**: 171.
 LEONARD, S. L., PERLMAN, P. L., and KURZROK, R. (1947).—*Proc. Soc. Exp. Biol.* **66**: 517.
 LEWIS, W. H., and WRIGHT, E. S. (1935).—*Carnegie Inst. Wash., Contrib. Embryol.* **25**: 113.
 LONG, J. A. (1912).—*Univ. Calif. Publ. Zool.* **9**: 105.
 McLEAN, D., and ROWLANDS, I. W. (1942).—*Nature* **150**: 627.
 MAYER, H. (1842).—*Arch. Anat. Physiol., Lpz.* p. 17.
 MENKIN, M. F., and ROCK, J. (1948).—*Amer. J. Obstet. Gynaec.* **55**: 440.
 MORICARD, R. (1949).—*C. R. Soc. Franç. Gynéc.* Dec. 226.
 MORICARD, R. (1950).—*Nature* **165**: 763.
 MORICARD, R., and BOSSU, J. (1949*a*).—*Bull. Acad. Méd. Paris* **133**: 659.
 MORICARD, R., and BOSSU, J. (1949*b*).—*Bull. Soc. Gynéc. Obstet. Paris* **1**: 30.
 NAGEL, W. (1888).—*Arch. mikr. Anat.* **31**: 342.
 ONANOFF, J. (1893).—*C. R. Soc. Biol. Paris* **45**: 719.
 PINCUS, G. (1930).—*Proc. Roy. Soc. B* **107**: 132.
 PINCUS, G. (1936).—"The Eggs of Mammals." (The Macmillan Co.: New York.)
 PINCUS, G. (1939).—*J. Exp. Zool.* **82**: 85.

- PINCUS, G., and ENZMANN, E. V. (1932).—*J. Exp. Biol.* 9: 403.
- PINCUS, G., and ENZMANN, E. V. (1934).—*Proc. Nat. Acad. Sci. Wash.* 20: 121.
- PINCUS, G., and ENZMANN, E. V. (1935).—*J. Exp. Med.* 62: 665.
- PINCUS, G., and ENZMANN, E. V. (1936).—*J. Exp. Zool.* 73: 195.
- PINCUS, G., and SHAPIRO, H. (1940).—*Proc. Amer. Phil. Soc.* 83: 631.
- ROCK, J., and MENKIN, M. F. (1944).—*Science* 100: 105.
- ROWLANDS, I. W. (1944).—*J. Endocrinol.* 3: 384.
- RUNNER, M. (1947).—*Anat. Rec.* 99: 564.
- SCHENK, S. L. (1878).—*Mitth. Embr. Inst. Univ. Wien* 1: 107.
- SOBOTTA, J. (1895).—*Arch. mikr. Anat.* 45: 15.
- SOBOTTA, J., and BURCKHARD, G. (1910).—*Anat. Hefte.* 42: 433.
- SODERWALL, A. L., and BLANDAU, R. J. (1941).—*J. Exp. Zool.* 88: 55.
- SWYER, G. I. M. (1946).—*Lancet* 251: 755.
- SWYER, G. I. M. (1947).—*Biochem. J.* 41: 413.
- TAFANI, A. (1889).—*Arch. Ital. Biol.* 11: 112.
- THIBAUT, C. (1947a).—*C. R. Soc. Biol. Paris* 141: 607.
- THIBAUT, C. (1947b).—*C. R. Acad. Sci. U.R.S.S.* 224: 297.
- THIBAUT, C. (1948).—*C. R. Soc. Biol. Paris* 142: 495.
- UMBAUGH, R. E. (1949).—*Amer. J. Vet. Res.* 10: 295.
- VAN BENEDIN, E. (1875).—*Bull. Acad. Belg. Cl. Sci.* 40: 686.
- WALLRAFF, J., and BECKERT, H. (1939).—*Z. mikr.-anat. Forsch.* 45: 510.
- WISLOCKI, G. B., BUNTING, H., and DEMPSEY, E. W. (1947).—*Amer. J. Anat.* 81: 1.
- YAMANE, J. (1930).—*Cytologia* 1: 394. (Cited by Pincus 1936.)
- YAMANE, J. (1935).—*Cytologia* 6: 233.

EXPLANATION OF PLATE 1

- Fig. 1.—Sperm mid-piece and tail projecting from the interior of the vitellus, through the zona, and out into the surrounding medium. In this egg the head of the sperm has already developed into a pronucleus. x1120.
- Fig. 2.—The slit in the zona considered to have been made by a sperm in entering the egg. The surface of the vitellus, at a lower focal plane, is seen as a blurred background. x1120.
- Fig. 3.—When the egg shown in Figure 2 was rolled beneath the cover-slip so that the slit came to the free surface of the egg, the vitelline material exuded as shown, thus demonstrating a break in the continuity of the zona. x1120.
- Fig. 4.—An egg from an immature rat with 23 sperms in the perivitelline space; the largest number seen in a rat egg. x700.

PENETRATION OF SPERM INTO THE MAMMALIAN EGG



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